NUTRIENT NICHES: AN INVESTIGATION OF NUTRITIONAL ECOLOGY IN A GENERALIST HERBIVORE COMMUNITY

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2014

Major Subject: Entomology

ABSTRACT

Understanding how diversity is maintained is a classic question in ecology. A diverse group of organisms can often be found utilizing the same resource. For example, in grasslands there are communities of grasshoppers containing many generalist species with overlapping diets that are likely competing for resources. To explore how species that overlap in host plant use can coexist, I investigated a recent hypothesis in nutritional ecology that species-specific macronutrient requirements in generalist insect herbivores could represent different nutrient niches. As a model system I used a community of grasshoppers in Central Texas.

First, I surveyed variation in plant macronutrient content and compared this data to the grasshopper community. By assaying levels of digestible protein and carbohydrate in abundant forbs and grasses at different sites, I produced a 'nutrient landscape' available to foraging herbivores and found significant correlations between plant nutrients and grasshopper abundance.

To further explore the role of plant macronutrient shifts in controlling grasshopper populations, I manipulated water availability in plots of grassland during a severe drought. Total grasshopper density and diversity were lower in water-stressed plots despite previous observations of drought-induced outbreaks. The effect of water stressed plants on grasshoppers depended on their diet, and how different plant groups responded to water stress.

I then compared host plant use to macronutrient requirements among 11 dominant grasshopper species. I found differences associated with functional diet groupings. I also found intake differences among mixed-feeders with highly overlapping diets, which could potentially represent nutrient niches.

Finally, I tested the nutrient niche hypothesis in a greenhouse competition experiment using three species of generalist grasshoppers with overlapping diets. I found mixed support for the nutrient niche hypothesis. Body size was more important for predicting competitive outcomes.

Understanding community-wide patterns of nutrient regulation in insect herbivores is in its infancy. While the plant nutrient landscape plays a large role in consumer populations, we are far from understanding how species-specific nutrient regulation differences might impact communities. Perhaps the potential effects of nutrient intake differences are inconsequential next to other ecological factors. Future comparative studies should determine what evolutionary factors shape nutrient requirements.

DEDICATION

I would like to dedicate this work to my parents Robert and Lilia Lenhart, and my grandparents Elmer Lenhart, Elva ‘Mickey’ Lenhart, Heriberto Alvarado, and Carmen Alvarado. If it were not for their hard work and support I would never have had the opportunities I have been given.

ACKNOWLEDGEMENTS

My research was generously supported by a number of groups. Texas A&M University initially supported me with a Diversity Fellowship. The Texas A&M Department of Entomology honored me with a Knipling-Bushland-SWAHRF Scholarship. I would like to thank the Orthopterists' society for entrusting me with a grant to begin my research. I received financial and logistical support from the Texas Ecolab grant through Braun and Gresham Attorneys at Law and the numerous Texas landowners who participate in this amazing program. I would also like to acknowledge the substantial support I have gotten from the USDA through the NIFA fellowship I was awarded (2012-67011-19930).

For their great support, guidance, mentorship, and friendship I want to sincerely thank my co-advisors Micky Eubanks and Spencer Behmer. They have worked hard to make me a better scientist. I also owe a debt of gratitude to my other committee members Raul Medina and William Rogers for their insights and suggestions. Additionally, the Eubanks and Behmer labs have provided me feedback on every stage of this research, assisted with sampling and experiments in the field and lab, and reviewed drafts of my manuscripts. My bright coworkers from the Eubanks lab have included Courtney Tobler, Steve Frank, Carolyn Zehnder, Shawn Wilder, Ricardo Ramirez, Loriann Garcia, Collin McMichael, Alison Bockoven, Warren Sconiers, Jared Ripple, Brittany DeLoach, Scott Bockoven, and Adrianna Szczepaniec. The brilliant colleagues I have had in the Behmer lab include Marion Le Gall, Carrie Deans, Rebecca

Clark, Lisa Ellis, Xiangfeng Jing, Steve Cook, Travis Gates, Karl Roeder, Andy Boswell, Sasha Kay, Angie Martin, and Harriet Thindwa. I am indebted to a hardworking army of undergraduate students in the Behmer and Eubanks lab that have made this work possible including Rheah Mcdonell, Alaina Nicolle Sheehy, Julie England, Hollis Geyer, Kimberly DeBlois, Amber Nava, Paola Aranda, Lauren McColley, Alba Mejorado, Hayley Jane Ask, Jason Gould, Britney Burback, and Karina Flores.

I would like to thank the staff of the Balcones Canyonlands National Wildlife Refuge including Deborah Holle, Scott Rowin, David Maple, Jim Mueller, and most especially Chuck Sexton. They granted me permits to work on the refuge, facilitated all my collecting and research trips, answered all questions I had, worked with me when it came to burning tracts, and I hope to work with them in the future.

Monique Reed was indispensable for help with plant identification and vouchering specimens. Fiona Clissold was a great collaborator and provided the excellent plant chemistry assays. Greg Sword provided great advice and the use of his lab's microscope for gut content analysis. David Ferguson tracked down the identity of enigmatic *Melanoplus* spp. Since my undergraduate days I have gotten essential advice from many people including Shawn Dash, Jose Pacheco, William Mackay, Jerry Johnson, Ian Kaplan, Nathan Burkett-Cadena, Brian Locke, Ken Stabinsky, Vicente Mata-Silva, Nick Lannutti, Justin Hobert, Hector Riveroll, and Paul Hotchkin. I have gotten a lot of crucial advice and support from all the magnificent members of the Texas A&M Entomology department and the Ecology and Evolutionary Biology program in

official settings and the occasional happy hour. I must also sincerely thank all my family and friends for supporting my ‘bug habit’ over the course of my life.

Finally I would like to thank my two greatest passions. First is my lovely girlfriend Marion Le Gall who has been my best friend and put up with me these last years. Second I want to acknowledge all the thousands of insects that gave their lives to help me learn more about their spectacular biology.

NOMENCLATURE

BCNWR	Balcones Canyonlands National Wildlife Refuge
C	Carbon
GF	Geometric Framework
IT	Macronutrient intake target
N	Nitrogen
p:c	Protein:carbohydrate ratio

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CHAPTER I

INTRODUCTION

1.1 Coexistence in Phytophagous Insects

Herbivorous insects include some of the most species rich and abundant taxa on the planet with estimates ranging from 400,000 to several million species (Schoonhoven et al. 2005). Understanding how this diversity is maintained is a classic question in ecology. A diverse group of herbivores can often be found utilizing the same plant or group of plant species. According to the competitive exclusion principle (Gause 1932) multiple species competing for the same resource cannot coexist if other ecological factors are constant. One competitor will always overcome the other due to even the slightest competitive advantage, leading to extinction or to an evolutionary or behavioral shift towards a different ecological niche. However, whether or not herbivorous insects in nature compete for food resources is a controversial question.

Classic competition theory predicts that two organisms engage in a struggle for limiting resources (Schoener 1982). These interactions are thought to become more intense with increasing density, spatiotemporal co-occurrence, ecological similarity, and phylogenetic relatedness. Coexistence therefore can only be achieved through divergence in resource use. The importance of interspecific competition in shaping communities was assumed early on in all organisms including phytophagous insects. This was established through observations of niche segregation which is thought to reduce or eliminate competition (Ross 1957, Ueckert and M. 1971, Le Quesne 1972,

Schoener 1974, Rathcke 1976, Waloff 1979, Connell 1980). Manipulative experiments were rare prior to the 1980's although classic works, such as the competition experiments with *Tribolium* (Park 1948) were performed. Even so, arguments against the importance of competition began to arise. Investigators discovered coexistence in species with extensive niche overlap, 'harmonious' coexistence with apparently no aggression, facilitation, numerous vacant niches, and unsaturated communities (Lawton 1982, Strong 1982, Lawton 1984). The fact that vegetation in the field is largely unconsumed was thought to mean that herbivores are not food limited. Instead it was concluded that herbivores are maintained at low densities by natural enemies (predators, parasites, pathogens) and thus are not in competition with each other (Hairston et al. 1960). Based on this accumulation of evidence, the possibility that interspecific competition among phytophagous insects was a negligible force influencing community structure gained popularity (Lawton and Strong 1981, Connell 1983, Schoener 1983, Lawton and Hassell 1984, Strong 1984, Strong et al. 1984, Jermy 1985).

In subsequent years, investigators responded by conducting controlled, manipulative experiments to investigate competition between phytophagous insects. These experimental studies were able to reestablish competition as a dominant force in herbivore communities. The review by Denno et al. (1995) found that among 193 pair-wise interactions in these studies, 76% demonstrated competition. However, this percentage varied between functional feeding guilds (sap sucking, chewing, galling, stem boring etc.). It was determined that sessile feeders were most likely to compete and free-living chewing insects were least likely. In addition, the authors determined that

often host plants, natural enemies and physical factors mediated competitive interactions. This topic was revisited in a meta-analysis of phytophagous insect competition studies by Kaplan and Denno (2007) which found that classic competition theory did not explain the interaction patterns observed. The hypothesis that competition was reduced between insect species that shared a host plant but were in different feeding guilds was found to be false. The meta-analysis determined that intra-guild competition was generally found to be equivalent to inter-guild competition. Also the expectation that when more leaf tissue is consumed competition between folivores intensifies was falsified. In addition, defoliation rates did not correlate with competition intensity. The study found mixed support for the effects of phylogeny and spatial/temporal separation on competition. There was also mixed results in the literature on whether the effects of intraspecific competition were less than or greater than the effects of interspecific competition. In general, Kaplan and Denno (2007) showed that species compete asymmetrically, meaning one species was usually a far superior competitor under the conditions studied. This meant that other factors must be suppressing the advantage of the superior competitor in nature to allow coexistence. The authors concluded that the lack of predictable effects of guild or species association, defoliation, phylogeny, and spatial/temporal separation are likely due to indirect competition through host plant or predator mediated interactions. Therefore an incorporation of other factors such as apparent competition (Holt 1977) and induced plant defenses (Karban and Myers 1989) into theories on herbivore competition is necessary. Some researchers have begun to investigate these complex interactions. For example, Smith et al. (2008) demonstrated

that coexistence between three aphid species is mediated by a combination of ant predation, ant mutualisms and plant genotype effects.

Despite these discoveries, most of the experiments analyzed in the literature focused on competition between multiple species of insects on one plant species. Factors explaining why competitive exclusion does not seem to occur between generalist herbivores with overlapping diets are even less understood.

Generalist herbivores fall into two broad categories: 'True generalist', that is, polyphagous herbivores in which an individual organism will forage for, browse, and graze on multiple plant species (Singer 2001); and 'Composite generalist', that is species recorded from multiple host plant species in different families, but in which a specific individual or host race only feeds on one or few of these species (Fox and Morrow 1981, Sword and Chapman 1994, Sword and Dopman 1999).True generalists switch host plants often and eat smaller amounts of each than specialists (Blust and Hopkins 1990). This diet mixing is known to improve survival and development time and/or reduce the variability or searching cost of food resources (Bernays and Minkenberg 1997, Hagele and Rowell-Rahier 1999, Singer 2001, Miura and Ohsaki 2004, Unsicker et al. 2008, Franzke et al. 2010). Improved performance by diet mixing can be accounted for either by optimizing nutritional intake from multiple, inferior foods (Pulliam 1975, Westoby 1978) or by diluting the effects of any one plant's defenses (Freeland and Janzen 1974). This behavior would largely mitigate any effects of plant defense or genotype on generalists. Therefore plant defense may not be a significant factor in coexistence.

Polyphagous herbivores make up a major component of many communities. A classic example is grasshoppers. Grasslands make up 40.5% of terrestrial landmass (White et al. 2000). In these ecosystems grasshoppers are the most abundant invertebrate herbivore and can severely reduce aboveground net primary production (Gibson 2009). In many grassland ecosystems 10 or more polyphagous grasshopper species can be found coexisting. In the grasslands of Texas there can be over 50 species (Appendix Table A.1). Many of these species have broadly-overlapping diets (Mulkern et al. 1969, Ueckert and M. 1971, Joern 1979a, Pfadt and Lavigne 1982, Joern 1985), co-occur in space/time at a small scale, are usually closely ecologically and phylogenetically related, and there is strong evidence that they compete (Ritchie and Tilman 1992, Belovsky and Slade 1995, Chase 1996a, Beckerman 2000, Liu et al. 2007). Even during frequent grasshopper outbreak scenarios, multiple coexisting species make up the community (Pfadt 1982, Watts et al. 1982). Therefore, coexisting grasshoppers should be under intense pressure to partition resources yet they seem to defy Gause's postulate (Gause 1932). Attempting to explain coexistence of generalists through interactions such as apparent competition or plant defenses is equally unsatisfactory. Predators reduce grasshopper population density but instead of promoting diversity, lower species richness has been observed under natural predation pressure (Joern 1986, 1992). There has been no investigation of the effects of induced plant chemical defenses on generalist grasshopper competition; however these species may circumvent many defenses by dilution (Freeland and Janzen 1974). Adaptation to specific induced plant defenses is assumed to be rare in grassland acridids (Anderson and Wright 1952, Gangwere 1961,

Mulkern 1967, Mulkern et al. 1969, Ueckert and M. 1971, Otte and Joern 1977, Uvarov 1977, Sheldon and Rogers 1978, Joern 1979a) and therefore may not be a major factor in maintaining diversity in acridid communities.

1.2 Nutrient Niche Hypothesis

A possible explanation for this paradoxical diversity is the nutrient niche hypothesis. Behmer and Joern (2008) demonstrated that coexisting polyphagous grasshoppers selectively feed to reach species-specific ratios of protein and carbohydrate known as nutrient intake targets. This could mean that these species have segregated niches based on nutrients rather than host plant taxa. In nature this could mean that certain species focus on plant tissues (from different plant species or individuals) that compliment their specific nutrient intake target. Resource-quantity limitations are usually thought of as a single currency such as prey items, host plants, biomass, nitrogen (N) or carbon (C) without linking these transfers with the many nutrients organisms required (Moe et al. 2005). Competition for nutrients is well known in a number of organisms. This has led to the idea that niche segregation can occur in nutritional dimensions. Nutrient niches have been proposed for plants (Tilman 1988, Paoli et al. 2006), gut microbes (Freter et al. 1983, Chang et al. 2004), and plankton (Petersen 1975, Yoshiyama et al. 2009). Kinnear et al. (1979b) took the niche width proposed by Hutchinson (1957) and applied it to multiple nutrient dimensions in a grazing herbivore. Kinnear et al. (1979) showed how ruminant-like mammals were able to expand their realized nutritional niche by utilizing symbiotic gut microbes. These mammals would

use pre-gastric fermentation to process plant tissue with low nutritional value and high fiber and cellulose. Clancy and King (1993) defined a nutritional niche for a conifer specialist caterpillar in terms of calcium, magnesium, and phosphorus using response surface design. Importantly this study demonstrated that the ratio of mineral nutrients was critical. Since these studies were conducted, a new method, the 'Geometric Framework', has been developed to investigate how an organism balances multiple nutrient needs in a variable nutritional environment (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993, Raubenheimer and Simpson 1994, Simpson and Raubenheimer 1995). Using this methodology, Behmer and Joern (2008) have proposed that generalist herbivores could be utilizing nutritional niches. Various grasshopper species could coexist by foraging for different plant species, individuals, or plant parts to meet well-defined ratios of protein and carbohydrate.

1.3 The Geometric Framework

The Geometric Framework (GF) is a state-space modeling approach that explores how animals attempts to solve the problem of balancing multiple and changing nutrient needs in a multidimensional and variable nutritional environment (Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993, Raubenheimer and Simpson 1994, Simpson and Raubenheimer 1995, 2012). It has features in common with other nutritional ecology models (i.e. resource allocation model (Tilman 1982) and ecological stoichiometry (Sterner and Elser 2002)), but differs in that it focuses on the physiology

and behavior of individuals. In addition, the GF provides a graphical way to interpret relationships between multiple nutrients consumed by organisms.

The GF treats an organism within a multidimensional nutrient space where there are as many axes as there are functionally relevant (fitness-affecting) nutrients. There is a mixture and blend of these nutrients that is optimal, the nutritional target (Simpson and Raubenheimer 2012). It is likely that animals have evolved a suite of behavioral and physiological mechanisms that enable them to approach this target (Raubenheimer and Simpson 1999, Simpson and Raubenheimer 2012). The position of this target can change over time depending on an organism's stage of development and the environmental circumstances. The GF has two additional targets. The intake target represents the amount of nutrients that an animal needs to ingest in order to reach its nutritional target since not all ingested nutrients are absorbed. There is also the growth target, which indicates the amount of nutrients incorporated into body tissues (nutritional target minus metabolic requirements).

An organism such as a generalist grasshopper can reach its intake target by eating from a range of different foods, regulating the amount of an individual food eaten, or through a combination of these two mechanisms (Simpson and Raubenheimer 2000). In graphical representations of the GF, foods are represented as trajectories, or 'rails', running through a defined nutrient space of pre-determined dimensions. Beginning at the origin, an organism moves along the rail as it consumes food defined by its nutritional composition. If that target lies on the food rail an organism is able to reach its intake target with limited decision making. The intake target cannot be reached if an organism

is restricted to one nutritionally unbalanced food, and it must make some kind of nutritional compromise. When foods are nutritionally imbalanced, conflicts may arise between the mechanisms regulating the intake of the nutrients (Raubenheimer and Simpson 1993). In this case our animal must employ some 'decision rule' such as overeating to obtain limiting nutrients.

A more typical situation for an organism is that it will have the opportunity to choose among foods with different nutritional profiles. When choosing between a nutritionally balanced and imbalanced food, the organism should always eat the former. When no nutritionally balanced food is available, an organism can still reach its intake target if nutritionally complementary foods are available and the organism eats from them in the correct proportions. Switching between different food items can occur at any time-scale, ranging from bites to days, and the rate at which switching occurs will be determined by the costs associated with such behaviors.

1.4 Evidence for the Nutritional Niche

The GF as well as other multiple lines of evidence from the literature theoretically support the possibility of nutrient niches:

1) Combinations of nutrients found within a plant are the basis of fitness in phytophagous insects. Generally the most limiting nutrient for an herbivore is thought to be available N (amino acids/protein) in a plant (Mattson 1980, White 1984, Mattson and Haack 1987, Ritchie and Tilman 1993, White 1993, Joern and Behmer 1998, Ritchie and Olff 1999, Ritchie 2000, DeGabriel et al. 2008). However, both protein and

carbohydrates have been shown to be important in insect herbivore nutritional decision making (Waldbauer et al. 1984, Telang et al. 2001, Lee et al. 2002, Lee et al. 2003, Berner et al. 2005, Thompson and Redak 2005, Despland and Noseworthy 2006, Lee et al. 2006, Merkx-Jacques et al. 2008). In some studies other micronutrients have been shown to play a more minor role in food selection (Stockhoff 1993, Simpson et al. 2006, Behmer 2009). Nevertheless, macronutrients seem to dominate the food selection choices of insect herbivores (Trumper and Simpson 1993, Bernays and Bright 2005, Thompson and Redak 2005, Behmer 2009). This means that food choice is a complex problem with multiple 'currencies' of plant quality, which different species of herbivores are trying to regulate their intake for.

2) Herbivores can feed selectively and tightly regulate ingestion of food for a ratio of macronutrients close to optimum (Waldbauer et al. 1984, Raubenheimer and Simpson 1993, Simpson and Raubenheimer 1993, Bernays and Chapman 1994, Raubenheimer and Simpson 1994, Simpson and Raubenheimer 1995, Chambers et al. 1996, Singer and Stireman 2001, Simpson and Raubenheimer 2012).

3) Predation risk makes could provide strong selective pressure to select food that is closest to the optimum macronutrient quality. In addition to physically killing herbivores, the very presence of a predator has been found to have strong effects on prey behavior. These predator indirect effects include reduced foraging, reduced feeding, and avoidance of predator occupied space which can include host plant shifts (Beckerman et al. 1997, Schmitz 1998b, Schmitz and Suttle 2001, Schmitz 2003, Schmitz et al. 2004, Schmitz 2008, Hawlena and Schmitz 2010, Hawlena et al. 2011). This increases the

pressure on an herbivore to feed on plant tissue which will most readily satisfy its nutritional needs (Abrams and Schmitz 1999) and may decrease the overlap in plant tissue which would be fed on by another species with a different nutrient intake target. Herbivores that can satisfy their nutritional needs effectively and with the least amount of competition with other herbivores should be spending minimal time vulnerable to predation while feeding and may therefore have a selective advantage.

4) Coexisting polyphagous herbivores are selectively feeding to reach species-specific protein-carbohydrate intake targets Behmer and Joern (2008). In practice this could mean multiple generalist species selectively feed on different plant species, individual plants, and specific plant parts that complement one another allowing the herbivores to reach their respective nutrient intake targets and avoid competition. While the seven species of *Melanoplus* whose nutrient intake targets were determined by Behmer and Joern (2008) each had non-overlapping targets, further studies need to be conducted to determine if this pattern is widespread.

5) Availability of essential nutrients varies greatly across time, space, and host plant (White 1978, Mattson 1980, Denno and McClure 1983, Mattson and Haack 1987, Louda and Collinge 1992, Rosenthal and Berenbaum 1992, White 1993, Bernays and Chapman 1994, Mole et al. 1994, Oedekoven and Joern 2000). This means the distribution and concentration of food nutrients available to a generalist herbivore, the 'nutrient landscape', can be very heterogeneous with many peaks and valleys of plant quality and abundance. This heterogeneity could provide multiple niches in 'nutrient space', which species could partition.

6) Natural selection can act on mechanisms of nutrient regulation. Nutrient regulation in insect herbivores, including intake targets, regulation of nutrient intake on imbalanced diets, and postingestive processes, are hypothesized to be shaped by natural selection (Raubenheimer and Simpson 1997, 1999, Simpson and Raubenheimer 1999, Raubenheimer and Simpson 2003, Behmer and Joern 2008, Simpson and Raubenheimer 2012). The ability for natural selection to act on these mechanisms was formally tested by Warbrick-Smith et al. (2006) when they reared populations of the diamondback moth, *Plutella xylostella*, for eight generations on carbohydrate-rich or carbohydrate -poor foods (artificial diet or an *Arabidopsis* mutant). On carbohydrate-rich foods the insects progressively evolved the ability to eat excess carbohydrate without storing it as fat, most likely due to a fitness cost for storing lipids. On carbohydrate-poor foods caterpillars had a greater propensity to convert ingested carbohydrate to fat and store it. The intake target was also observed to shift slightly (Warbrick-Smith et al. 2006), but in this specialist metabolic adaptation was the dominant response.

1.5 Study Questions

The main focus of my dissertation was to test the existence of nutrient niches. That is, do differences in species-specific nutrient requirements affect communities of generalists? Specifically, I investigated three questions: 1) Are changes in grasshopper community assemblages and population density correlated to heterogeneity in the plant nutrient landscape? 2) Do coexisting generalist grasshopper species have unique nutrient

intake targets? 3) Do levels of interspecific competition correlate to differences in nutrient intake targets between species?

CHAPTER II

NUTRIENT LANDSCAPES AND INSECT HERBIVORES: A MULTI-YEAR FIELD STUDY LINKING PLANT PROTEIN-CARBOHYDRATE CONTENT WITH GRASSHOPPER POPULATIONS

2.1 Overview

Plant nutrient quality is an important parameter when considering bottom-up effects on herbivores. Advances in nutritional ecology have consistently determined that animals actively regulate for specific ratios of protein:carbohydrate (p:c) through foraging decision making. Many of these studies rely on artificial diets and data describing the multidimensional variation in plant protein and carbohydrate content across time, space, and plant taxa in the field are virtually non-existent. I document temporal and spatial variation in the digestible protein and nonstructural carbohydrate content of forbs and grasses in a Central Texas grassland during the summer of 2009 and 2010. I then used a model selection approach to detect correlations between herbaceous plant macronutrient content and grasshoppers, the system's dominant insect herbivore. I produced a graphical representation of the 'nutrient landscape' available to generalist grassland herbivores. The ratio of plant p:c was surprisingly carbohydrate biased with significant differences between forbs and grasses, between sampling times, and between sites. Grasshopper densities were correlated with different nutritional metrics based on year. During a severe drought in 2009 the variation in plant protein and carbohydrate shrank over the course of the summer, which was correlated to a decline in both total and

grass-feeding grasshopper densities. During the wetter summer of 2010, spatial variation in all grasshopper densities were negatively correlated with plant protein and p:c ratio, challenging the nitrogen limitation hypothesis. My results suggest that grasshopper population dynamics are tied to plant macronutrient content, but these relationships may vary between years.

2.2 Introduction

Plant nutrient quality is an important parameter when considering bottom-up effects on herbivores (White 1983, Awmack and Leather 2002, Codron et al. 2007, Behmer 2009). However, plant quality is a complex factor to define and has been assessed in many different ways. To estimate quality for grazing livestock, variables such as total nitrogen (N), crude protein, digestible organic matter, total carbohydrates, water content, ash, cell wall content, and phosphorous are measured (Huston et al. 1981, Mengel and Kirkby 2001). In ecological literature total N, total carbon (C), and total carbohydrates, often represented as 'energy', are the standard (Sterner and Elser 2002, Anderson et al. 2004). However, with the exception of certain mineral salts (Trumper and Simpson 1993, Simpson et al. 2006, Kaspari et al. 2008, Dudley et al. 2012), animals do not consume elements in their pure chemical forms (Simpson and Raubenheimer 2012). Instead, animals acquire most of their N and C in macronutrients such as protein, amino acids, digestible carbohydrates, and lipids (Sterner and Elser 2002, Raubenheimer et al. 2009, Simpson and Raubenheimer 2012). When analyzing the nutritional content of plants to investigate interactions with herbivores, proxies for

digestible protein and carbohydrates may not be sufficient. When total N is estimated instead of digestible protein, deleterious N-based defensive compounds are incorporated into the estimate (Schoonhoven et al. 2005, Joern et al. 2012). At least one study has suggested that when herbivore selection of host plants is compared to total N versus available N, significant correlations can only be made with available N (DeGabriel et al. 2008). In addition, estimating total C or total carbohydrates includes indigestible cellulose and lignin in both, and toxic C-based defensive compounds in the former (Raubenheimer et al. 2009). Therefore, *digestible* protein and carbohydrates should be estimated.

Not only should studies of plant quality focus on the actual content of digestible macronutrients, but the blend must also be taken into account. A growing body of literature has found that the ratio of protein:carbohydrate (p:c) is of critical importance when animals make foraging decisions (Raubenheimer and Simpson 1993, Behmer 2009, Raubenheimer et al. 2009, Simpson and Raubenheimer 2012). Using a technique termed the 'Geometric framework', investigators can consider an organism within a nutrient space where axes are functionally relevant macronutrients (Simpson and Raubenheimer 2012). Within this nutrient space the technique allows us to identify an 'intake target' representing the amount of nutrients the animal actively regulates for by adjusting the amount of an individual food eaten, eating different foods, or combining both. Investigators using this method have demonstrated macronutrient intake targets in many taxa including humans, other mammals, birds, fish, slime molds, and insects (Raubenheimer and Simpson 1997, Simpson and Raubenheimer 2001a, Simpson and

Batley 2003, Behmer 2009, Felton et al. 2009, Dussutour et al. 2010). A majority of the work with the geometric framework has used insect herbivores as a model system (Behmer 2009, Simpson and Raubenheimer 2012). However a major drawback of the geometric framework is its heavy reliance on artificial diets to find nutrient intake targets and understand the choices an herbivore makes when presented with different quality foods (Raubenheimer et al. 2009). Furthermore, data describing the multidimensional nutrient landscape of plant protein and carbohydrate content across time, space, and plant taxa in the field are virtually non-existent. This can impede the ability of researchers to apply findings from artificial diet experiments to organisms in nature.

In this study, I document natural temporal and spatial variation in the digestible protein and nonstructural carbohydrate content of forbs and grasses in a Central Texas grassland. I then used a model selection approach to detect correlations between herbaceous plant macronutrient content and the dominant insect herbivore in the ecosystem: acridid grasshoppers. Grasshoppers were the focus of this study for several reasons. First, investigators using the geometric framework have found that grasshoppers tightly regulate for specific ratios of p:c, and these nutrients are thought to play a large role in their host plant selection (Chambers et al. 1995, Simpson et al. 2002, Clissold et al. 2006, Behmer and Joern 2008, Fielding and Defoliart 2008, Boswell 2009, Goeriz Pearson et al. 2011, Parsons 2011, Cease et al. 2012). Second, they are the dominant insect herbivores in grassland systems (Gibson 2009). Densities can reach 20+ individuals/m^2 in many grassland ecosystems worldwide (Uvarov 1977, Fielding and Brusven 1993, Barrientos 1995, Kooyman et al. 1997). The grasshopper community in

most grasslands contains species that represent three distinct functional feeding groups with different diet breadths: forb-feeders, grass-feeders, and mixed-feeders (Chapman and Sword 1997). Alpha diversity of grasshopper communities can be high with 10-30+ species in a single locality (Otte 1976, Joern 1979b, 1985, Cigliano et al. 2000, Branson 2010). Finally, grasshopper density and community structure fluctuate significantly across space and time and may reflect differences in food quality (Jonas and Joern 2007, Loaiza et al. 2011, Joern et al. 2012).

Using this system I asked two main questions: 1) What is the 'nutrient landscape' available to a foraging generalist herbivore, and how does it vary between grasses and forbs, over the course of a growing season, and between sites? 2) Can variation in the nutrient landscape be linked to intra-seasonal temporal or spatial differences in grasshopper densities in terms of total density and the different functional feeding groups? If so, what is more important: protein content, nonstructural carbohydrate content, macronutrient ratio, or the variance of any of these nutrient metrics? Higher variance in macronutrient content among plants could be important because it would allow a broader nutrient space for a consumer to regulate intake in (Raubenheimer and Simpson 1999, Behmer and Joern 2008). This study provides, for the first time, a comprehensive quantification of the macronutrient landscape available to a community of foraging herbivores. It also gives a natural context to the growing literature on macronutrient regulation using artificial diets (Behmer 2009, Simpson and Raubenheimer 2012) and identifies which macronutrient variables may be most

predictive of population density for this group of ecologically and economically important insect herbivores.

2.3 Methods

2.3.1 Study system

This study was conducted at the Balcones Canyonlands National Wildlife Refuge (BCNWR) located northwest of Austin, Texas. The refuge covers parts of Burnet, Williamson and Travis Counties. The geology of the study site is characteristic of the Edwards plateau with limestone hills and shallow rocky soils. The BCNWR (established in 1992) is not grazed by livestock and is managed with prescribed burns on a 2-4 year cycle. The grasshopper community is diverse with 56 species of grasshoppers (Orthoptera: Acrididae) and includes widespread species of western North America and the Great Plains as well as several Texas endemics.

During June-August, 2009 and June-September, 2010, 26 sites (Table 2.1) were sampled for grasshopper density, grasshopper community composition, plant nutrient content, and plant biomass. Sites were 60 m × 60 m and were located in areas of mixed-grass prairie and oak (*Quercus* sp.) savannah. The maximum distance between any two sites was 9 km and the closest was 0.07 km. My sampling in 2009 was designed to quantify temporal variation in the plant nutrient landscape while sampling in 2010 emphasized spatial variation. In 2009, the same four sites were sampled on June 4, July 2, and August 19. In 2010, I used 14 different sites with 3 sites sampled on June 25 and 11 sites in September 2-16.

Table 2.1 Sites used in Chapter II with geographic coordinates as well as descriptions of the habitat and plant community. Site names correspond to names of land tracts on the Balcones Canyonlands National Wildlife Refuge. Exotic grass was mainly *Bothriochloa ischaemum* var. *songarica* and in some cases *Sorghum halepense*.

Sample year	Site name	Latitude	Longitude	Habitat	Plant community
2009	Flying X 1	30°38'7.79"N	98° 5'12.16"W	Open grassland	Exotic grass dominated
	Flying X 2	30°38'3.52"N	98° 5'0.64"W	Open grassland	Exotic grass dominated
	Mullen 3	30°39'50.81"N	98° 4'2.82"W	Open grassland	Exotic grass dominated
	Mullen 4	30°40'4.91"N	98° 4'5.63"W	Open grassland	Exotic grass and *Grindelia*
2010	Gainer 5	30°37'38.71"N	97°59'55.95"W	Oak savannah	Exotic grass dominated
	Gainer 6	30°37'51.69"N	97°59'57.40"W	Rocky open grassland	Native calcareous grassland
	Gainer 7	30°37'11.90"N	97°59'44.76"W	Open grassland	Exotic grass dominated
	Gainer 8	30°37'48.16"N	97°59'53.33"W	Open grassland	Native grassland
	Gainer 9	30°37'51.40"N	98° 0'0.15"W	Rocky open grassland	Native calcareous grassland
	Flying X 10	30°37'41.59"N	98° 4'59.11"W	Rocky grassland	Exotic grass dominated
	Beard 11	30°38'12.66"N	98° 4'30.29"W	Oak savannah bottomland	Native grassland
	Doeskin 12	30°37'18.41"N	98° 4'23.23"W	Open grassland	Native calcareous grassland
	Doeskin 13	30°37'7.47"N	98° 4'13.57"W	Open grassland hillside	Native calcareous grassland
	Mullen 14	30°39'29.28"N	98° 3'40.98"W	Oak savannah	Exotic grass and weedy forbs
	Beard 15	30°38'15.34"N	98° 4'22.82"	Oak savannah bottomland	Native grassland
	Doeskin 16	30°37'11.55"N	98° 4'27.88"W	Open grassland	Native calcareous grassland
	Flying X 17	30°38'4.42"N	98° 5'24.11"W	Open grassland	Exotic grass and weedy forbs
	Beard 18	30°38'8.99"N	98° 4'15.75"W	Rocky oak savannah	Native calcareous grassland

2.3.2 Grasshopper sampling

Overall grasshopper density at each site was sampled using the standardized ring technique (Onsager and Henry 1977, Joern 2005) whereby all the grasshoppers within the 0.1m² area of a wire ring left in the field are counted. Twenty rings were placed randomly along four 60 m transects per site with at least 2m between rings. Rings were marked with survey flags and left undisturbed for >2h before counting to allow natural redistribution of any flushed grasshoppers. After grasshopper density was recorded, I sampled grasshopper community composition at each site by systematic sweep netting using a 38.1cm diameter sweep net. Four parallel sweep transects of 80 sweeps were made within each site and grasshoppers collected were frozen and subsequently sorted in

the laboratory. I identified unknown grasshopper nymphs by collecting similar nymphs in the field and rearing them in the laboratory to adulthood. Sweep netting allows reliable estimates of relative abundance of grasshopper species (Evans et al. 1983). I estimated the density of individual species by multiplying the relative abundance of individual species by the mean grasshopper density at a site averaged across the four transects. Using diet studies in the literature (Joern 1979a, Capinera and Sechrist 1981, Joern 1983, 1985, Richman et al. 1993, Pfadt 2002) as well as an analysis of gut content for a subset of local grasshoppers (Chapter IV), I assigned species to functional feeding groups: forb-, grass-, and mixed-feeding species. Mixed-feeding species consume both grasses and forbs.

2.3.3 Plant nutrient content analysis

I clipped leaves from three individuals of most abundant (visually estimated) three forb species and three grass species found within each site. These samples were placed in paper envelopes and flash frozen in the field using liquid nitrogen for nutrient content analysis in the laboratory. Frozen samples were subsequently lypholized and green foliar plant material removed and ground to a fine powder using a Wiley cutting mill (size 20 mesh). Total nonstructural carbohydrates and soluble protein were analyzed using the methodology of Clissold et al. (2006). Protein was extracted from 20 mg samples with 500μL 0.1M NaOH by sonication for 30 min and heating at 90°C for 15 min. Samples were centrifuged (13,000 rpms for 10min), the supernatants were removed, and the pellet washed with 300μL of 0.1M NaOH and centrifuged again. After

removing this supernatant and combining it with the previous supernatant, the pH was neutralized using 11µL of 5.8M HCl. Protein was then precipitated with 90µL of 100% trichloroacetic acid. The samples were centrifuged to form a pellet of protein that was quickly washed with 100µL of -20°C acetone after the supernatant was removed. The acetone was allowed to evaporate and proteins were re-suspended in 1mL of 0.1M NaOH and then diluted to ensure the concentration of NaOH were less than 0.01M so that it did not interfere with Coomassie blue solution used by the Bradford assay. To quantify digestible protein I used the Bio-Rad micro assay based on the Bradford assay (Bradford 1976) with 0–8 µg of IgG (bovine gamma globulin) as the standard with duplicate samples read in triplicate. Total non-structural carbohydrates were extracted from 20 mg samples placed for 1 h in a boiling water bath with 1 mL 0.1M H_2SO_4 and determined colourimetrically (0–75 mg (D +) glucose standard) using the phenol–sulphuric acid assay (Dubois et al. 1956). Total digestible protein and nonstructural carbohydrates were calculated as the percent of dry plant weight.

2.3.4 Plant biomass

Vegetation biomass was estimated by averaging clipped vegetation from eight 0.1 m^2 plots of vegetation at each site clipped at ground level. The eight plots were randomly located along the four transect (two per transect) established for grasshopper density counts. Vegetation was sorted to living grass and forb, dried for 48 h at 55°C in a drying oven, and weighed to estimate total biomass for each functional plant group (dry weight in g/0.1m^2).

2.3.5 Statistical analysis

I investigated how foliar macronutrient content among herbaceous grassland plants varied between functional plant groups, over time, and across sites. I evaluated these sources of variance using MANOVA because macronutrient levels in plants are not independent of one another. I report the Wilks' lambda test statistic. I examined how protein and carbohydrate content varied across a growing season (June-August 2009), between the functional plant groups, and the interaction of these factors. In this analysis sites was used as a blocking factor. Finally I focused on the impact site variation has (2010 samples) on the protein and carbohydrate content of grasses and forbs. To evaluate variance within a significant factor I used specified contrasts using likelihood-ratio tests (SAS Institude Inc. 2012) with Bonferonni corrected values of α. I also tested for differences in total grasshopper density over space and time. With the 2009 grasshopper densities I used the repeated measures MANOVA approach (Wilks' Lambda test statistic) to test for significant effects of sites, month, or a site × month interaction. With the 2010 grasshopper density dataset I used ANOVA to test for significant differences across sites. MANOVA and ANOVA were conducted in JMP 10 (JMP 1998-2007)

To determine which variables (month, plant macronutrient contents, plant macronutrient ratio, or plant biomass) best explained grasshopper density I used multiple linear regression analysis and a model selection approach with Akaike's Information Criterion adjusted for small sample size (ΔAIC_c). This analysis is well suited for correlative inference using observational data in complex field systems (Burnham and

Anderson 2002, Johnson and Omland 2004). Model selection for 2009 and 2010 datasets were conducted separately since sampling regimes varied. Eight variables were compared in the 2009 models: month, mean plant protein, mean plant carbohydrate, plant protein coefficient of variance, plant carbohydrate coefficient of variance, mean plant p:c ratio, plant p:c ratio coefficient of variance, and plant biomass. All of these variables with the exception of month were compared in 2010 models since sampling was not longitudinal. I included variables describing the variance in plant nutrient content since the breadth of 'nutrient space' could be important for herbivores actively regulating for a particular nutrient ratio (Raubenheimer and Simpson 1999). Within each year, model selection was conducted separately for total grasshopper density as well as forb-, grass-, and mixed-feeding grasshopper estimated densities. Only grass nutrient content and biomass were included in the analysis of grass-feeding grasshopper density and only forb nutrient content and biomass were included in models for forb-feeding density. Biomass and nutrient content for both grass and forbs combined were used for analyses of mixed-feeder and overall grasshopper density. For each year and grasshopper density combination I fit all possible models and calculated: the information theoretic criterion corrected for small sample size (AIC_c), the model's AIC_c difference compared with the best model's AIC_c (Δw), the normalized AIC_c weight which are interpreted as the relative likelihood of the model given the data (w_i), the evidence ratio which is interpreted as the likelihood that the model is the best (ER), the model's adjusted R^2, and the model's significance (P) (Burnham and Anderson 2002). I used linear regression analyses to determine the relationship between response and

explanatory variables identified by the best-fit and competing models. My criteria for the best fit and competing models were those with w_i >0.1 (Joern et al. 2012). All model selection and linear regression was conducted using JMP 10 (JMP 1998-2007).

2.4 Results

2.4.1 Foliar nutrient landscape

Concentrations (mean % dry weight ± 1 SE) of digestible protein and nonstructural carbohydrate from 462 individual grassland plants sampled from June-September of 2009 and 2010 across 18 sites are shown in Fig. 2.1. The only plants having a p:c ratio >0.8 were among individuals of five aster species (*Ambrosia psilostachya*, *Melampodium leucanthum*, *Ratibida columnifera*, *Gutierrezia texana*, *Verbesina virginica*) and a spurge (*Croton monanthogynous*). Protein and carbohydrate content of the 38 plant species sampled is summarized in Table 2.2. I found that protein and carbohydrate content in 2009 (Fig. 2.2) varied significantly between sites (MANOVA site: Approx. $F_{6,400} = 15.80$, $P < 0.001$*), across months (MANOVA month: Approx. $F_{4,400} = 8.06$, $P < 0.001$*), between grasses and forbs (MANOVA plant functional group: $F_{2,200} = 57.02$, $P < 0.001$*), and co-varied across month and plant functional group (MANOVA month × plant functional group: Approx. $F_{4,400} = 10.96$, $P < 0.001$*). Forbs appear to simultaneously decrease in carbohydrate content and slightly increase in protein content across all three months (Fig. 2.2; June forbs vs. July forbs, $F_{2,200} = 8.91$, $P < 0.001$*; July forbs vs. August forbs, $F_{2,200} = 8.76$, $P < 0.001$*). Grasses retained the same p:c content between the first two months but increased in carbohydrate

content between July and August (Fig. 2.2; June grass vs. July grass, $F_{2,200}$= 0.59, *P* =0.557; July grass vs. August grass, $F_{2,200}$= 16.86, *P* <0.001*). In samples from 2010

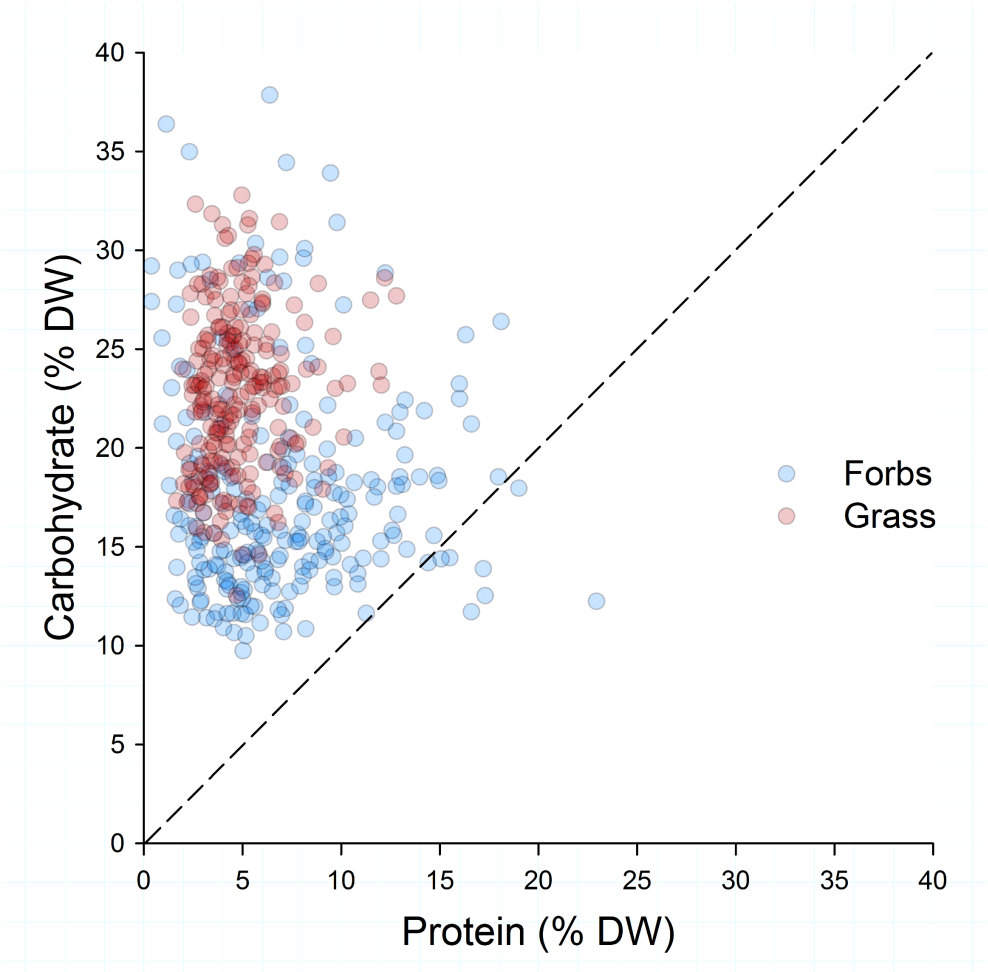

Fig. 2.1 The macronutrient landscape (digestible protein and nonstructural carbohydrate) of forbs and grass from the Balcones Canyonlands National Wildlife Refuge. Data points represent individual plants from the three most abundant forbs (blue) and grass (red) species (visually estimated, 3 individual plants per species) from 12 samples (4 sites across 3 months) in 2009 and 14 sampled sites in 2010. Macronutrient content was measured as % dry weight. The dotted line represents a balanced 1:1 protein:carbohydrate ratio.

(Fig. 2.3) protein and carbohydrate content again varied significantly between sites (MANOVA site: Approx. $F_{26,446} = 11.28$, $P < 0.001$*), between grasses and forbs (MANOVA plant functional group: $F_{2,223} = 76.59$, $P < 0.001$*), and co-varied across site and plant functional group (MANOVA site × plant functional group: $F_{26,446} = 4.18$, $P < 0.001$*).

Table 2.2 Macronutrient content of individual plant species sampled during 2009 and 2010 at the Balcones Canyonlands National Wildlife Refuge.

Plant family	Plant species	n	Protein (% DW)	SE (% protein)	Carbohydrate (% DW)	SE (% carbohydrate)	Protein:Carbohydrate ratio	SE (p:c ratio)
Asteraceae	*Ambrosia psilostachya*	18	7.06	0.69	14.54	0.63	0.4787	0.0371
	Amphiachyris dracunculoides	6	5.08	0.39	15.01	0.38	0.3389	0.0260
	Gaillardia pulchella	6	2.64	0.40	12.56	0.23	0.2091	0.0296
	Grindelia squarrosa	9	3.98	0.66	14.51	0.95	0.2992	0.0669
	Gutierrezia texana	15	11.05	0.73	16.13	0.41	0.7002	0.0585
	Liatris mucronata	15	5.19	0.40	16.23	1.94	0.3845	0.0447
	Melampodium leucanthum	12	10.54	1.23	19.53	0.55	0.5366	0.0592
	Ratibida columnifera	9	8.09	0.57	13.84	0.45	0.5838	0.0351
	Solidago sp.	3	6.32	0.38	28.05	0.49	0.2250	0.0110
	Tetraneuris linearifolia	6	6.25	0.69	15.09	0.47	0.4149	0.0452
	Verbesina virginica	9	12.63	1.72	13.99	0.43	0.9320	0.1527
	Wedelia hispida	9	6.69	0.58	15.16	1.01	0.4430	0.0285
Euphorbiaceae	*Croton monanthogynous*	42	10.27	0.64	18.87	0.66	0.5664	0.0398
	Tragia ramosa	21	1.92	0.23	21.91	1.28	0.1043	0.0184
Gentianaceae	*Centaurium beyrichii*	3	7.63	0.92	35.41	1.24	0.2174	0.0321
Lamiaceae	*Monarda citriodora*	3	3.14	1.01	15.90	0.13	0.1971	0.0627
	Salvia texana	3	4.98	0.07	11.07	0.98	0.4573	0.0417
	Scutellaria sp.	6	2.98	0.39	15.85	0.84	0.1862	0.0205
Scrophulareaceae	*Agalinis heterophylla*	6	5.48	0.77	26.15	2.42	0.2060	0.0148
	Verbascum thapsus	3	3.75	0.38	20.48	1.02	0.1857	0.0275
Smilaceae	*Smilax bona-nox*	3	12.96	0.51	18.82	1.35	0.6968	0.0631
Verbenaceae	*Phyla nodiflora*	6	3.24	0.28	12.99	0.51	0.2497	0.0195
	Verbena bipinnatifida	6	6.80	1.59	28.62	0.96	0.2397	0.0566
	Verbena canescens	12	4.73	0.40	18.12	0.78	0.2621	0.0187
Poaceae	*Aristida sp.*	9	6.84	0.55	23.36	1.43	0.3070	0.0368
	Bothriochloa ischaemum var. songarica	60	4.42	0.22	24.26	0.50	0.1836	0.0087
	Bothriochloa laguroides torreyana	18	4.59	0.27	22.72	1.19	0.2142	0.0192
	Bothriochloa saccharoides	12	8.02	1.14	23.93	0.85	0.3274	0.0409
	Bouteloua curtipendula	21	4.07	0.26	24.39	0.72	0.1721	0.0146
	Bouteloua hirsuta	15	3.61	0.21	21.60	1.22	0.1714	0.0120
	Bromus sp.	3	3.63	0.50	20.97	1.26	0.1767	0.0324
	Eragrostis sp.	6	2.65	0.15	21.84	1.47	0.1255	0.0139
	Muhlenbergia reverchonii	6	2.72	0.19	17.55	0.22	0.1550	0.0106
	Nassella leucotricha	24	4.24	0.20	23.36	0.95	0.1918	0.0156
	Panicum sp.	3	4.03	0.19	23.29	2.20	0.1747	0.0095
	Schizachyrium scoparium	27	5.63	0.30	21.57	0.43	0.2624	0.0135
	Sorghum halepense	15	5.39	0.44	22.12	1.00	0.2420	0.0156
	Sporobolus asper	6	7.72	0.46	20.45	0.79	0.3837	0.0366

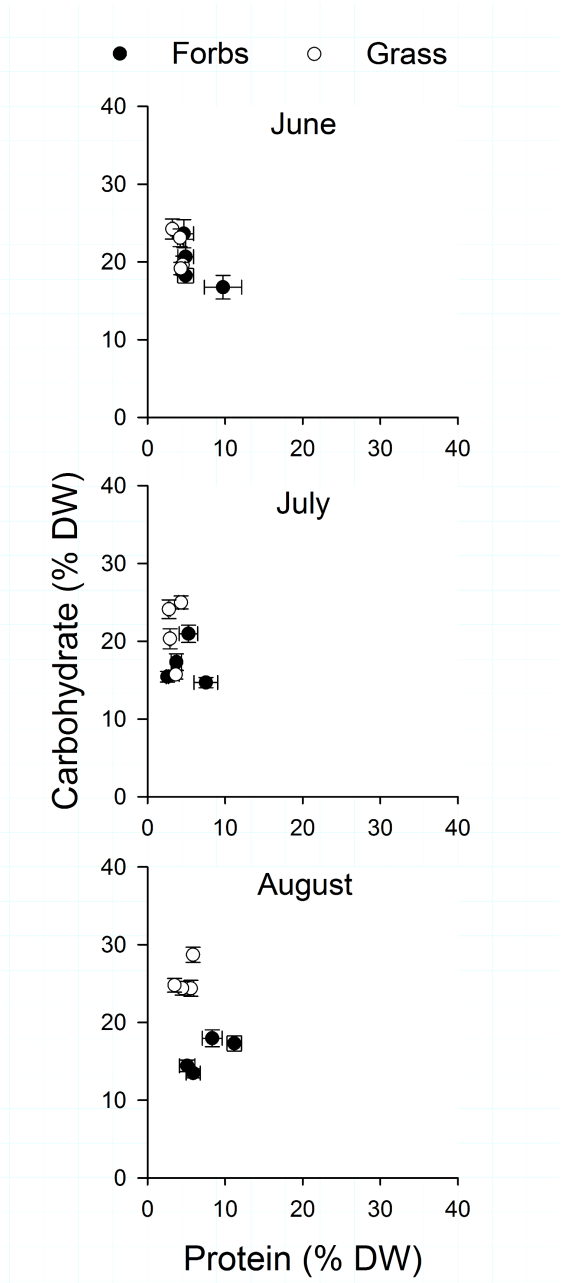

Fig. 2.2 Macronutrient content of grasses and forbs across four sites repeatedly sampled in June, July, and August 2009. The digestible protein and nonstructural carbohydrate was measured as % dry weight and is presented as mean ±1 SE.

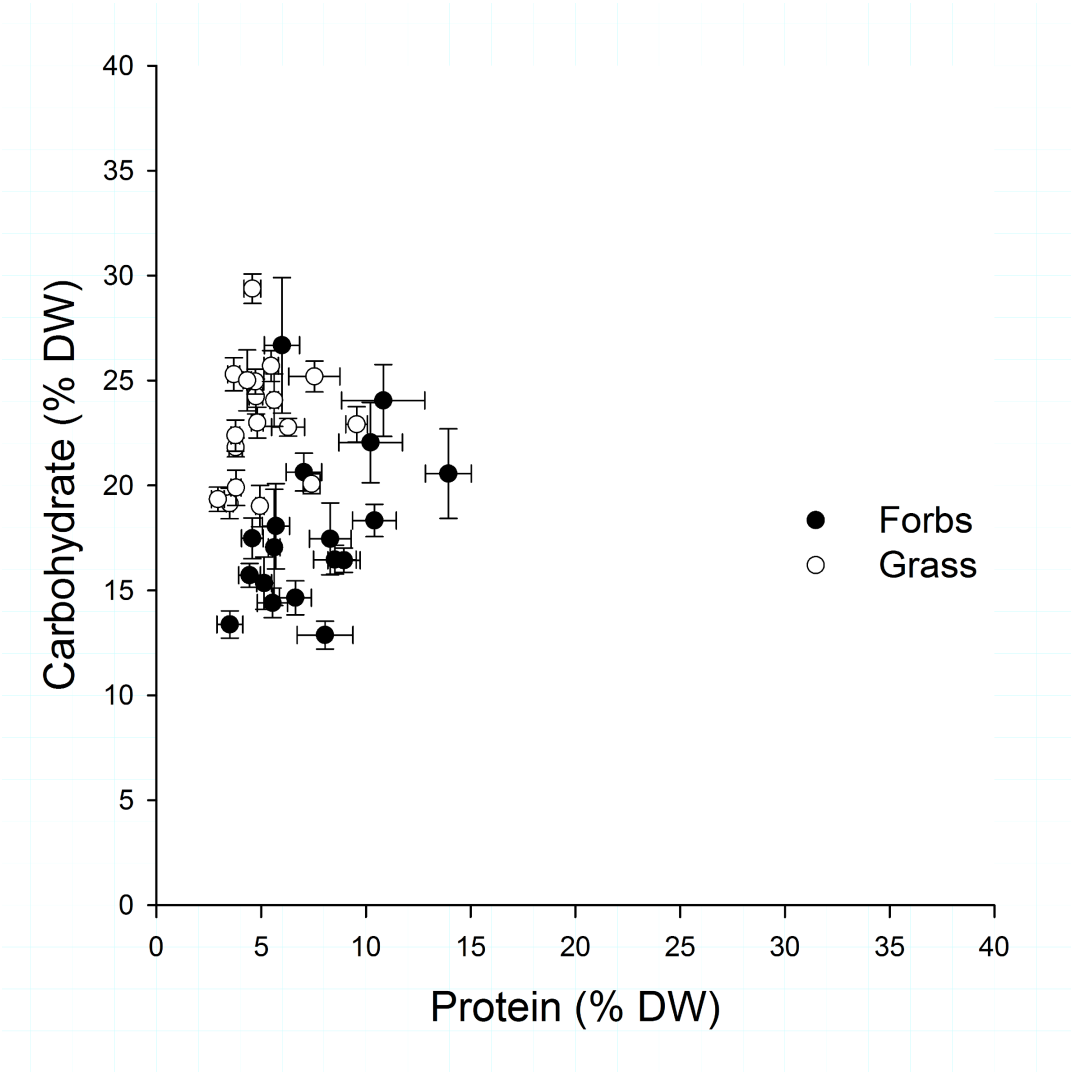

Fig. 2.3 Macronutrient content of grasses and forbs across 14 sites sampled in 2010. The digestible protein and nonstructural carbohydrate was measured as % dry weight and is presented as mean ±1 SE.

2.4.2 Overall grasshopper density

Overall grasshopper densities in 2009 and 2010 across sites are shown in Fig. 2.4. In 2009, density was generally low with an average of 1.7 ± 0.2 grasshoppers/m^2. I found significant variation in grasshopper density across months (repeated measures MANOVA month: $F_{2,11} = 8.24$, $P = 0.007$*), but no significant difference between sites (repeated measures MANOVA site: $F_{3,12} = 0.48$, $P = 0.700$) or any interaction of month and site (repeated measures MANOVA month × site: Approx. $F_{6,22} = 1.05$, $P = 0.419$). Overall grasshopper density was similar in June and July, but decreased in August to an average 0.7 ± 0.3 individuals/m^2 (Fig. 2.4; June vs. July, $F_{1,45} = 1.84$, $P = 0.182$; July vs. August, $F_{1,45} = 7.35$, $P = 0.009$*). In 2010 grasshopper density averaged 5.7 ± 0.5 grasshoppers/m^2. During this year density varied significantly among sites (Fig. 2.4; ANOVA site: $F_{13,42} = 6.39$, $P < 0.001$*) with a roughly sevenfold difference that ranged from 1.5 to 11 individuals/m^2.

Using AIC_c I regressed grasshopper density across all taxa with plant macronutrient content, macronutrient ratio, and plant biomass to identify which models best fit the data (defined as w_i values > 0.1). For the 2009 dataset one model met my criteria (Table 2.3) and was significant at $\alpha = 0.05$. Overall grasshopper density in 2009 was best predicted by month, and the coefficient of variance for plant p:c content. Grasshopper density in this year had a significant positive relationship with month ($R^2 = 0.66$, $F_{1,10} = 1.27$, $P = 0.001$) and plant p:c variance ($R^2 = 0.38$, $F_{1,10} = 6.26$, $P = 0.031$). Among the 2010 comparisons, two models met my criteria (Table 2.4) but were only marginally significant ($0.05 < P < 0.1$). Overall grasshopper density in 2010 was best

predicted by plant p:c ratio and plant protein which both had marginally significant negative relationships with overall grasshopper density.

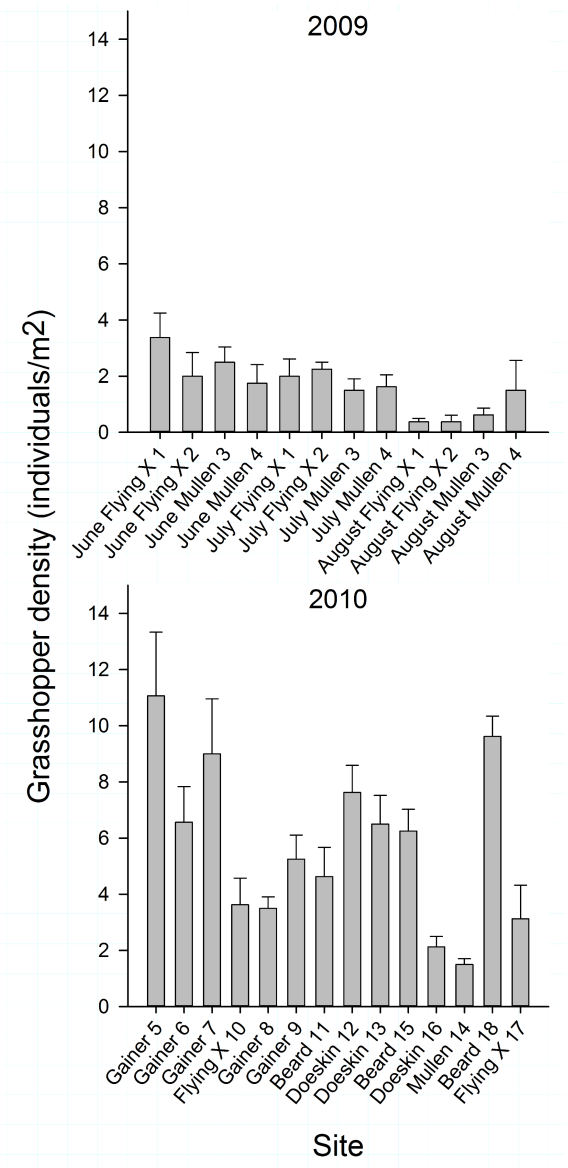

Fig. 2.4 Overall grasshopper densities at each sampling date/site in 2009 and each site in 2010. Densities (mean +1 SE) were estimated using the standardized ring count method (Onsager and Henry 1977, Joern 2005).

Table 2.3 Summary of results identifying the best sets of models to predict grasshopper density in 2009. Models were selected for all species (a), and each functional feeding group (b-d) using Akaike information theory criteria over 4 sites sampled monthly from June-August of 2009 on the Balcones Canyonlands National Wildlife Refuge. All possible multiple regressions were performed but only models that generated *wi* values of >0.1 are shown here (See the Methods section for more details).

Model	AICc	Δ*w*	*wi*	ER	Adj. R^2	*P*	Explanatory variables
a) All species							
	21.04		0.45	1	0.81	<0.001*	Month, Plant p:c ratio variance
b) Forb-feeding species							
	13.96		0.15	1	0.33	0.068	Month, Forbs carbohydrate
c) Grass-feeding species							
	22.38		0.24	1	0.82	<0.001*	Grass protein variance, Grass carbohydrate variance, Grass p:c ratio variance
	22.57	0.20	0.22	1.10	-0.09	0.733	Grass p:c ratio
d) Mixed-feeding species							
	-1.19		0.15	1	0.14	0.122	Plant p:c ratio variance
	-0.72	0.47	0.12	1.27	0.11	0.156	Plant biomass

Table 2.4 Summary of results identifying the best sets of models to predict grasshopper density in 2010. Models were selected for all species (a), and each functional feeding group (b-d) using Akaike information theory criteria over 14 sites sampled in 2010 on the Balcones Canyonlands National Wildlife Refuge. All possible multiple regressions were performed but only models that generated *wi* values of >0.1 are shown here (See the Methods section for more details).

Model	AICc	Δ*w*	*wi*	ER	Adj. R^2	*P*	Explanatory variables
a) All species							
	72.20		0.14	1	0.22	0.054	Plant p:c ratio
	72.34	0.14	0.13	1.07	0.21	0.057	Plant protein
b) Forb-feeding species							
	40.79		0.15	1	0.24	0.042*	Forb protein
	41.49	0.70	0.11	1.42	0.35	0.037*	Forb carbohydrate variance, Forb p:c ratio
	41.58	0.79	0.10	1.48	0.35	0.039*	Forb protein, Forb carbohydrate variance
c) Grass-feeding species							
	42.95		0.29	1	0.30	0.025*	Grass protein
	44.43	1.49	0.14	2.10	0.22	0.052	Grass p:c ratio
d) Mixed-feeding species							
	64.40		0.13	1	0.01	0.312	Plant biomass
	64.91	0.51	0.10	1.29	-0.03	0.438	Plant p:c ratio

2.4.3 Grasshopper functional feeding group density

The relative abundance of each functional feeding group at each site in 2009 and 2010 is shown in Fig. 2.5. I also applied the AIC_c approach to grasshoppers based on their functional feeding group. Only one model met my criteria for predicting densities of forb-feeders in 2009 (Table 2.3), but was marginally significant. Neither of the variables in this model were significantly correlated in independent linear regressions. In 2010, forb-feeders density were best predicted by forb protein and competing models included forb carbohydrate variance, and forb p:c ratio (Table 2.4). Forb feeder density in this year had a significant negative relationship with forb protein (R^2=0.30, $F_{1,12}$= 5.18, *P*=0.042) and a marginally significant negative relationship with forb p:c ratio (R^2=0.24, $F_{1,12}$= 3.70, *P*=0.079).

AIC_c identified two models (Table 2.3) that met my criteria for grass-feeder density in 2009 with one being significant at *P*<0.05. Grass-feeder density in 2009 was proportionally high (Fig. 2.5) and had a marginally significant positive relationship with grass protein variance (R^2=0.27, $F_{1,10}$= 3.75, *P*=0.082) and a significant positive relationship with grass carbohydrate variance (R^2=0.51, $F_{1,10}$= 10.41, *P*=0.009). In 2010 grass-feeder density was correlated with two models (Table 2.4) which included grass protein and p:c ratio. Grass-feeder density in this second year of the study had a significant negative correlation to grass protein content (R^2=0.35, $F_{1,12}$= 6.54, *P*=0.025), and a marginally significant negative relationship with grass p:c ratio (R^2=0.28, $F_{1,12}$= 4.67, *P*=0.052).

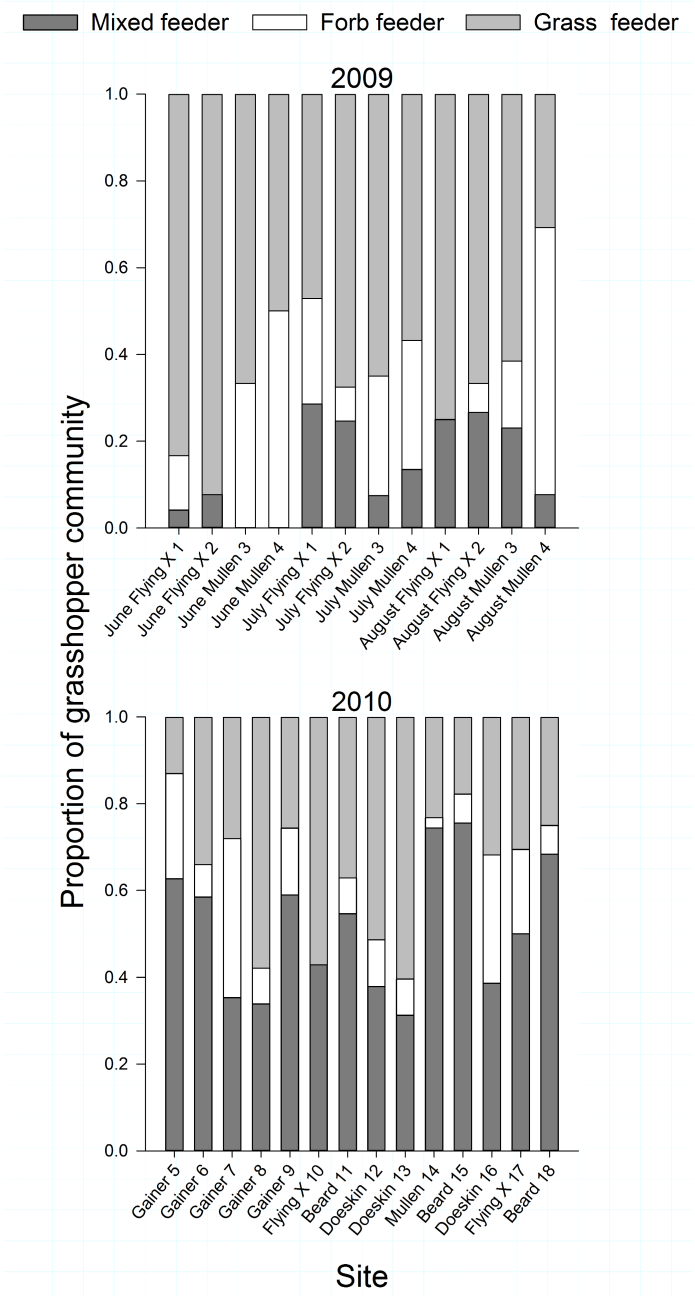

Fig. 2.5 Grasshopper community composition in 2009 and 2010. Relative proportions of grass-, forb-, and mixed-feeding grasshoppers are given for each sampling date/site in 2009 and each site in 2010. Community composition was calculated using relative abundance in sweep net samples (See methods section).

Mixed-feeder density in 2009 was relatively low (Fig. 2.5) and was predicted by two models that met my criteria but neither was significant (Table 2.3). In 2010 samples, mixed-feeders made up a relatively large part of the grasshopper community (Fig. 2.5). AIC_c identified two models which best-fit 2010 mixed-feeder density but, again, neither was significant (Table 2.4).

2.5 Discussion

I provide, for the first time, an accurate estimate of the macronutrient landscape available to a foraging generalist herbivore. Prior to this study, the nutrient landscape shape was thought to be very broad encompassing many carbohydrate- and protein-biased tissues (Raubenheimer and Simpson 1999, Raubenheimer et al. 2009, Behmer and Joern 2012). This was based on elemental content estimates (Mattson 1980, Poorter and Bergkotte 1992, Arendonk and Poorter 1994, Anderson et al. 2004). In general, the range of available protein:carbohydrate (p:c) contents I found in grasslands plants at the BCNWR was narrower in terms of ratio variation. The nutrient landscape was very carbohydrate biased with an average p:c of 1:2.4 in forbs and 1:4.7 in grasses. Only a few plants approached or exceeded a balanced 1:1 ratio (Fig. 2.1). These were exclusively forbs including several members of the Asteraceae and a Euphorbiaceae. Previous predictions of the nutrient landscape were correct when it came to grasses being more carbohydrate-biased than forbs (Behmer and Joern 2012). However, analysis of this herbaceous plant community found that the range of grass macronutrient content

was included within that of forbs, and grasses had a higher average absolute amount of carbohydrates than forbs.

The distribution of the nutrient landscape has important implications for the study of herbivore p:c regulation. Based on the data compiled from laboratory artificial diet experiments, most insect herbivore species studied actively regulate for around a 1:1 p:c ratio (Behmer 2009), although some species vary to the carbohydrate- or, more commonly, the protein-biased sides of nutrient space. In general these studies used artificial diets with high total macronutrient concentrations (40+%) and extreme protein-biased p:c ratios, (Simpson and Raubenheimer 1993, Chambers et al. 1995, Lee et al. 2002, Simpson et al. 2002, Behmer and Joern 2008), which, at least among plants included in my sampling, do not exist in nature. The assumption is often made among nutritional ecologists that nutrient requirements are in some way adapted to the content of food (Raubenheimer and Simpson 1997, 1999, 2003, Joern and Mole 2005, Behmer 2009). It would seem then that either: 1) this nutrient landscape is representative of plants in other habitats and many insect herbivores are consistently nutrient limited, over-ingesting carbohydrate to acquire protein (Berenbaum 1995, Berner et al. 2005); 2) these Texas upland calcareous grassland plants have a lower protein content and more carbohydrate-biased p:c ratio than plants in other habitats and the herbivores regulate for an equally carbohydrate-biased p:c intake (Chapter IV); or 3) there are large disparities between the total available nutrient content of a mechanically ground plant and what an herbivore is able to extract from actual host plant tissue using its mandibles and digestive enzymes (Zanotto et al. 1993, Clissold et al. 2006, Clissold et al. 2009,

Clissold et al. 2010). It is likely that, in fact, all three of these explanations are in play. To gain a full picture of the nutritional ecology of a particular organism, investigators will need to directly quantify the requirements of their target organism as well as the total and digestible nutrient content of the food under natural conditions.

The importance of nutrients for animals is undeniable, so it is critical to make assessments of plant quality available to an associated community of insect herbivores. Levels of protein and carbohydrates are well known to affect insect herbivore performance and diet selection in both artificial and natural environments (White 1978, Mattson and Haack 1987, Raubenheimer 1992, Slansky Jr et al. 1993, White 1993, Yang and Joern 1994, Joern and Behmer 1997, 1998, Behmer et al. 2001). Having documented the natural variation in the grassland plant macronutrient content, my next objective was to investigate which nutritional variables correlated with intra-seasonal temporal and spatial heterogeneity in both overall grasshopper density, and at the functional feeding group level (forb-, grass-, and mixed-feeder). In both years various plant nutrient content metrics were significantly correlated with grasshopper abundance, while host plant biomass was only selected for models of mixed-feeder density and was not significantly correlated independently. This could mean that these generalist herbivores are not limited by plant quantity, but rather by tissue of sufficient quality.

I found that nutritional variables that were predictive of grasshopper density differed substantially between years. The most striking pattern is that in 2009, all the significant plant nutrient variables had a positive correlation with density, while in 2010 all plant variables had negative correlations. It must be noted though that the sites and

timing of sampling differed between years. In 2009, total grasshopper abundance decreased across months (Fig. 2.4) along with a decrease in the breadth of p:c ratios available (Table 2.3). Grass feeder density also decreased as variation in the grass protein and carbohydrate content decreased. This decreasing variation in plant nutrient content is visible in the decreasing length of error bars around site/plant group means in Fig. 2.2. This means that as time passed, the nutrient space that a grasshopper could utilize to try and reach its optimal nutrient intake (Chapter IV) shrank. This pattern was likely due to the severe drought experienced in the area during that year. Rainfall from June-August of 2009 totaled 2.2 cm based on a weather station at the BCNWR and the Palmer Modified Drought Index was less than -3 throughout the sampling period, indicating severe drought (National Oceanic and Atmospheric Administration's National Climatic Data Center http://www.ncdc.noaa.gov/temp-and-precip/drought/historical-palmers.php). Although it has been suggested that drought-induced plant water stress is beneficial to insect herbivores and can lead to outbreaks (White 1969, Mattson and Haack 1987) I instead found evidence that drought may decrease the breadth of the nutrient landscape available to generalist herbivores. Without experimentally manipulating water availability, though, this requires further confirmation. I can, however, demonstrate that the macronutrient content of grasses and forbs shifted differently across months (Fig. 2.2). Whether this is a result of normal phenological shifts during the growing season (McNeill and Southwood 1978) or the severe drought is unknown, but it appears to have had consequences for the herbivores that specialize on these plant groups.

Spatial heterogeneity in plant macronutrient content (Fig. 2.3) during 2010 yielded a different set of predictive nutrient variables. In 2010 the BCNWR had received more rainfall (30cm between June and September) and had a Palmer Modified Drought Index >-2 throughout the sampling period, indicating 'mid-range' conditions (National Oceanic and Atmospheric Administration's National Climatic Data Center http://www.ncdc.noaa.gov/temp-and-precip/drought/historical-palmers.php). Overall grasshopper density as well as the density grass- and forb-feeders had a significant (or marginally significant) negative correlation with host plant protein content and p:c ratio. This seems counterintuitive considering the nitrogen-limitation hypothesis. It is often suggested that N is a limiting nutrient to many insect herbivores, including grasshoppers (McNeill and Southwood 1978, Mattson 1980, White 1993, Ritchie 2000, Awmack and Leather 2002, Matsumura et al. 2004, Bishop et al. 2010). However, not all studies show herbivore density responses to increased plant N (Strauss 1987, Kytö et al. 1996, Joern and Behmer 1998, Cease et al. 2012, Joern et al. 2012). Animals have been found to be unwilling to over-ingest protein (Simpson and Raubenheimer 2009), and high protein consumption can actually reduce performance (Clissold et al. 2006, Lee et al. 2008). A similar negative relationship with plant N was found in the locust *Oedaleus asiaticus* in Inner Mongolia (Cease et al. 2012). In that study, overgrazing was linked to lowered plant N that promoted outbreaks of the grass-feeding locust. A similar phenomenon could be operating in this grassland, but since I did not find a negative relationship with protein in 2009, the pattern may only be restricted to certain conditions such as non-

drought years. Further work such as fertilization studies will need to be done to determine the mechanisms responsible for this relationship.

Obviously the limiting factors for population size and a community's assembly rules cannot be completely explained by a reductionist approach which focuses only on macronutrients (Raubenheimer et al. 2009). This system is highly complex with many species interacting. The factors which determine grasshopper density and community structure are therefore going to be even more complex and likely include predator-mediated effects (Holt 1977, Belovsky and Slade 1993, Schmitz 2005), competition effects (Ritchie and Tilman 1992, Belovsky and Slade 1995, Chase 1996a, Kaplan and Denno 2007), dispersal and landscape effects (With and Crist 1995, J Haynes and T Cronin 2006, Haynes et al. 2007), as well as abiotic factors (Ritchie 2000, Skinner and Child 2000). Likely even more important are the species-specific traits of different host plants (Bernays and Chapman 1994). However, my study takes a significant step forward by describing the natural macronutrient space available to a group of well-studied model herbivore in nutritional ecology (Behmer 2009, Simpson and Raubenheimer 2012). My findings reinforce that nutrient content varies over time and space, and found that important nutritional predictors of abundance vary by functional feeding group and year. This approach to cataloging resource quality could be very informative when coupled with detailed information on the nutrient regulation of the target organism. With enough theoretical and empirical progress it will be possible to integrate information on the changing nutrient landscape into predictive models (Raubenheimer et al. 2009, Kearney et al. 2010, Simpson et al. 2010). By remotely

sensing for plant nutrient content (Foley et al. 1998, Zengeya et al. 2013) and other plant characteristics such as water content (Ullah et al. 2012b), biomass (Ullah et al. 2012a), and plant diversity (Gould 2000) there could be real world applications to the nutrient landscape. For example the nutrient landscape could be informative for rangeland management by improving predictive models of when grasshoppers and locusts may become pests and allowing targeted pest management (Branson et al. 2006, Behmer and Joern 2012). My study provides valuable quantitative data to begin parameterizing models. Far from being limited to rangeland grasshoppers, quantifying the available nutrient landscape could have predictive potential for any number of systems were organisms practice diet mixing and active nutrient regulation.

CHAPTER III

WATER STRESS IN GRASSLANDS: DYNAMIC RESPONSES OF PLANTS AND INSECT HERBIVORES

3.1 Overview

Global climate change is altering precipitation patterns. The effect of water stress on plant-herbivore interactions is poorly understood even though this is a primary ecological interaction that will be altered by climate change. This is especially true for grasslands where water is often limiting. In this study I manipulated water inputs in open grassland plots (1 m2) during a severe drought and assessed plant and insect herbivore responses. There were two watering treatments: ambient and supplemented. Supplemented plots received water weekly in amounts that mimicked average seasonal rainfall. For plants, I was interested in how water input affected protein and digestible carbohydrate content; previous studies predicted water stress would increase the concentration of these two nutrients. Grasshoppers are the dominant insect herbivores in grasslands, and I assessed their responses to water inputs by measuring abundance and diversity. Previous studies suggested grasshoppers would prefer water-stressed plots. Protein and digestible carbohydrate content in bulk grass and forb samples, plus plant biomass and diversity, were measured monthly (May-August). Immediately prior to harvesting plant tissue, I counted and identified individual grasshoppers in each plot. Grass biomass decreased with water stress, while grass macronutrient content and species diversity were unaffected. Water-stressed forbs were less protein biased relative

to watered forbs. Forb diversity was lower in water-stressed plots, but biomass was similar between treatments. Surprisingly, total grasshopper abundance and diversity were lower in water-stressed plots. However, grasshopper-feeding biology mattered. Mixed-feeders and grass-feeders had lower densities in water-stressed plots; forb-specialists showed no difference. This study reveals the manner in which water stress affects plant macronutrient content, abundance, and diversity in a grassland ecosystem, and its key insect herbivores. My results demonstrate the importance of focusing on plant and insect herbivore functional groups and provide valuable new data for models exploring the effects of global climate change.

3.2 Introduction

Global climate change is predicted to dramatically alter precipitation patterns and increase the frequency with which plants will be water stressed (Knapp et al. 2008, Dai 2010). This will likely have strong effects on associated herbivores, but despite high interest and years of study the effects of water stress on plant-herbivore interactions are still poorly understood (Huberty and Denno 2004). It has been suggested that water stress is beneficial to insect herbivores (White 1969, Mattson and Haack 1987). The proposed mechanism is that when plants are water stressed their quality to herbivores increases due to higher nutrient concentrations (White 1984, Brodbeck et al. 1987, Behmer and Joern 2012). However, water stress can constrain plant growth and decrease total nutrient content via decreased uptake of soil nutrients, decreased turgor pressure, xylem cavitation, reduced photosynthesis, senescence, and dieback of roots and shoots

(Hsiao 1973). In some plants, allelochemical concentrations can also increase under water stress (Inbar et al. 2001). Many of these water-stress responses can vary as a function of plant photosynthetic pathway, growth form, species, and genotype (Chaves et al. 2002), but under prolonged or severe water stress plants eventually die as a result of carbon starvation as well as hydraulic and symplastic failure (McDowell et al. 2008).

From an herbivore's perspective water stress induces changes in plant diversity (intraspecific variation in drought tolerance), quantity (changes in plant structure and biomass), and quality (shifts in nutrient concentration and allocation, reduced water content, and altered defensive chemistry) can all affect foraging and performance (White 1969, Koricheva et al. 1998, Huberty and Denno 2004). Plants differ in their tolerance of water stress (Chaves et al. 2002) and mortality of some species (McDowell et al. 2008) can lead to decreased diversity. High plant diversity might be particularly important for generalist herbivores because it provides greater opportunity to achieve a balanced nutrient intake via diet mixing (Bernays et al. 1994, Raubenheimer and Simpson 1999). When water is limiting, plant growth can be constrained (Hsiao 1973), limiting the quantity of plant food available to an herbivore. Plant macronutrient content, including protein and digestible carbohydrates (henceforth carbohydrate), is particularly critical for insect herbivores (Behmer and Joern 2012) which are known to simultaneously regulate their protein and carbohydrate intake (reviewed by Behmer 2009). Despite the known importance of these two key functional macronutrients, data describing the multidimensional nutrient landscape of plant protein and carbohydrate content across time, space, and plant taxa, let alone water gradients, are virtually non-existent.

In this study I tracked the effects of water inputs in open field plots ($1m^2$) on grassland plants and grasshoppers, the dominant grassland invertebrate herbivore. Water availability can affect multiple traits in plants but I was particularly interested in macronutrient content, due to the increasing emphasis on nutrition in insect herbivore foraging decision-making (reviewed by Behmer 2009). I quantified actual levels of protein and carbohydrate in field collected plants across multiple time points in the growing season. In addition, I recorded two other potential bottom-up factors that can affect herbivore density and diversity: plant biomass and plant diversity. Each time plant data was collected I recorded the abundance of individual grasshopper species without destructive sampling. A key aspect of my study is that the open plots allowed the mobile grasshoppers to self-distribute; this allows the grasshoppers to tell us which plots are preferred. Additionally, because I identified individuals to the species level, I could evaluate distributions in light of functional feeding groups (grass specialists, forb specialists, and mixed-feeding grasshoppers). Based on previous work (White 1984, Mattson and Haack 1987, Franzke and Reinhold 2011), I hypothesized that macronutrient content of grasses and forbs would be higher under water stress while plant biomass and diversity could decrease. In turn, I hypothesized that higher grasshopper abundance and diversity would be found on unwatered plots due to these plants having higher nutritional value. My study provides the first analysis of how drought affects macronutrient content of native grassland plants coupled with responses of grasshoppers, the key insect herbivores in my system.

3.3 Methods

3.3.1 Study system

Grasslands are an important ecosystem in which to study the effects of water stress because they make up ~ 40% of terrestrial landmass, experience frequent drought, and support most of the world's agriculture (Gibson 2009). This study was conducted at the Balcones Canyonlands National Wildlife Refuge (BCNWR) located northwest of Austin, Texas. The refuge covers parts of Burnet, Williamson and Travis Counties. The geology of the study site is characteristic of the Edwards plateau with limestone hills and shallow rocky soils. The experiment utilized areas of mixed-grass prairie and oak (*Quercus* sp.) savannah. The BCNWR (established in 1992) is not grazed and is managed with prescribed burns on a 2-4 year cycle. Samples were collected between 8 May and 4 August 2011. Site locations are given in Table 3.1. This grasshopper community is diverse with 56 species of grasshoppers (Orthoptera: Acrididae) and includes widespread species of the Great Plains as well as several Texas endemics (Appendix Table A.1). The grasshopper community is dominated by polyphagous mixed-feeding grasshoppers (eating both forbs and grasses) and, to a lesser extent, grass specialists. Forb specialists make up the smallest proportion of the community. Plant and grasshopper communities were similar across all sites.

Table 3.1 Site locations for experimental plots used in Chapter III. This table includes coordinates, approximate elevation, habitat descriptions, and the most recent year each site was burned.

Site	BCNWR Tract name	GIS coordinates for corner plots	Elevation (meters)	Habitat description	Most recent burn
1	Gainer	30° 37.786 N, 97° 59.899 W 30° 37.817 N, 97° 59.885 W 30° 37.819 N, 97° 59.888 W 30° 37.800 N, 97° 59.899 W	360.1	Loamy/ rocky soil, short grassland dominated by *Bouteloua hirsuta, Aristida* sp.	2009
2	Russell	30° 40.625 N, 98° 5.074 W 30° 40.608 N, 98° 5.053 W 30° 40.611 N, 98° 5.049 W 30° 40.628 N, 98° 5.066 W	399.5	Rocky soil, oak savannah dominated by *Erioneuron pilosum*, *Aristida* sp., *Schizachyrium scoparium*	2010
3	Beard	30° 38.185 N, 98° 4.237 W 30° 38.192 N, 98°4.255 W 30° 38.178 N, 98° 4.241 W 30° 38.190 N, 98° 4.260 W	380.0	Rocky soil, oak savannah hillside dominated by *S. scoparium*	??
4	Flying X	30° 37.855 N, 98° 4.784 W 30° 37.854 N, 98°4.763 W 30° 37.864 N, 98° 4.786 W 30° 37.865 N, 98° 4.772 W	415.6	Rocky shallow soil, sparse mixed grassland dominated by *S. scoparium*, *Bouteloua rigidiseta*, *Bothriochloa ischaemum* var. *songarica*	2006

3.3.2 Experimental protocol

From May to August of 2011 I conducted a water manipulation to examine the effects of drought on a diverse grasshopper community. This time period covers the lifecycle of most of the area's grasshopper fauna. I delimited four sites on the BCNWR with 28 $1m^2$ plots each. Plots were arranged in 2-3 rows and marked with survey flags. Plots were randomly assigned to two treatments: either supplemented with water from May to August or maintained as un-manipulated ambient controls. Control plots were the drought treatment and were allowed to desiccate during the La Niña-driven severe drought (based on predictions by the National Weather Service's Climate Prediction

Center, http://www.cpc.ncep.noaa.gov/). The la Niña-driven drought of 2011 was the worst single year drought in recorded Texas history (Nielsen-Gammon 2011). From September 2010 to September 2011 the BCNWR received 21.26 cm of rainfall, while the average annual rainfall since 1996 was 81.03 cm with peaks in May/June and September/October based on a weather station at the BCNWR. I supplemented watered plots at a rate intended to mimic the average pattern of rainfall in the area. Watering occurred weekly and was limited to 2.5cm of simulated rainfall per plot (25 L/m^2); by the end of sampling in August, watered plots had been supplemented with a total of 30 cm of simulated rainfall. Ambient rainfall during this same period was limited to 12.98 cm. I watered during early morning to allow infiltration and avoid excessive evaporation. Plots had a minimum distance of 1m between them to minimize run-off effects.

I took an initial sample of 4 control plots at each site the first week of May before watering began. After watering treatments had been established, I randomly sampled 8 plots per site (4 control, 4 watered) during the 1st week of June, July, and August. I did not re-sample individual plots from month to month and water manipulations ceased after a plot had been sampled. When sampling I quantified the grasshopper species richness and plot density as well as plant functional group species richness, biomass, and macronutrient content.

3.3.3 Grasshopper and plant sampling

Just prior to harvesting plant material, I counted and identified all grasshopper species present in each plot by flushing them by hand. Grasshoppers were not collected to avoid changing the grasshopper community at each site. Focusing on grasshoppers at the family level can reveal interesting general patterns, but this approach also obscures the biological reality that not all grasshopper species of the same family are equivalent. During data analysis, I also grouped species into their functional diet groups: forb-, grass-, and mixed-feeders (Appendix Table A.1). I measured plant species richness within a 0.25m^2 quadrat placed within each plot. Vouchers of plant species were deposited in the Texas A&M University Tracy Herbarium.

3.3.4 Plant biomass and nutrient content

To quantify drought-induced changes in the biomass of plant functional groups and their respective macronutrient content, I clipped a 0.1 m^2 area of vegetation within each plot at ground level. I separated living forbs and grasses from each sample because rangeland grasshoppers rarely consume dead dry litter. Samples were then lypholized, weighed, and subsequently milled and homogenized using a Wiley cutting mill (size 20 mesh). From these milled samples of forb and grass, replicated 20mg subsamples were taken for protein and carbohydrate analysis.

Total nonstructural carbohydrates and soluble protein were analyzed using the methodology of Clissold et al. (2006). Protein was extracted from 20 mg samples with 500µL 0.1M NaOH by sonication for 30 min and heating at 90°C for 15min. Samples

were centrifuged (13,000 rpms for 10min), the supernatants were removed, and the pellet washed with 300μL of 0.1M NaOH and centrifuged again. After removing this supernatant and combining it with the previous supernatant, the pH was neutralized using 11μL of 5.8M HCl. Protein was then precipitated with 90μL of 100% trichloroacetic acid. The samples were centrifuged to form a pellet of protein that was quickly washed with 100μL of -20°C acetone after the supernatant was removed. The acetone was allowed to evaporate and proteins were re-suspended in 1mL of 0.1M NaOH and then diluted to ensure the concentration of NaOH were less than 0.01M so that it did not interfere with Coomassie blue solution used by the Bradford assay. To quantify digestible protein I used the Bio-Rad micro assay based on the Bradford assay (Bradford 1976) with 0–8 μg of IgG (bovine gamma globulin) as the standard with duplicate samples read in triplicate. Total non-structural carbohydrates were extracted from 20 mg samples placed for 1 h in a boiling water bath with 1 mL 0.1M H_2SO_4 and determined colourimetrically (0–75 mg (D +) glucose standard) using the phenol–sulphuric acid assay (Dubois et al. 1956).

3.3.5 Statistical analysis

The four sites used were treated as blocks in all analyses. Site, month, watering treatment, and month*treatment interactions were used as explanatory variable for grasshopper plot density and species richness among all grasshoppers and for each individual feeding group (forb-, grass-, and mixed-feeders) in a Generalized Linear Model (GLM). Within the GLM a Poisson distribution was used as the count data were

not normally distributed (O'Hara and Kotze 2010). Within-group contrasts, i.e. between months or treatments, were made using likelihood-ratio tests (SAS Institude Inc. 2012). Because of the importance of the protein and carbohydrate ratio in insect herbivore nutrition (Behmer 2009) I analyzed protein and carbohydrate together using MANOVA against the same explanatory variables as above with arcsine transformed data. Plant macronutrient content was quantified in terms of percent dry mass. The Roy's Greatest Root test statistic is reported as most of the variance occurred in terms of protein. Effects of site, month, watering treatment, and month*treatment interactions on species richness and plant functional group biomass were analyzed using ANOVA for a randomized complete block design. All analyses were conducted in JMP 10 (SAS Institute, Inc.).

3.4 Results

Results for grass and forb responses, including effects on macronutrient content, biomass, and diversity, are presented first, followed by the effect of water manipulations on grass-specialist, forb-specialist, and mixed-feeding grasshoppers.

3.4.1 Grass responses

Protein and carbohydrate levels in grasses were unaffected by watering treatment (Fig. 3.1a-c, Table 3.2a). The protein-carbohydrate profile of grasses, however, varied significantly over the course of the growing season and between sites (Fig. 3.1a-c, Table 3.2a). Grass protein content decreased from June to July, ($F_{1,84}$=27.86, P<0.001), but then increased from July to August (Fig. 3.1b; $F_{1,84}$=17.13, P<0.001). Grass protein

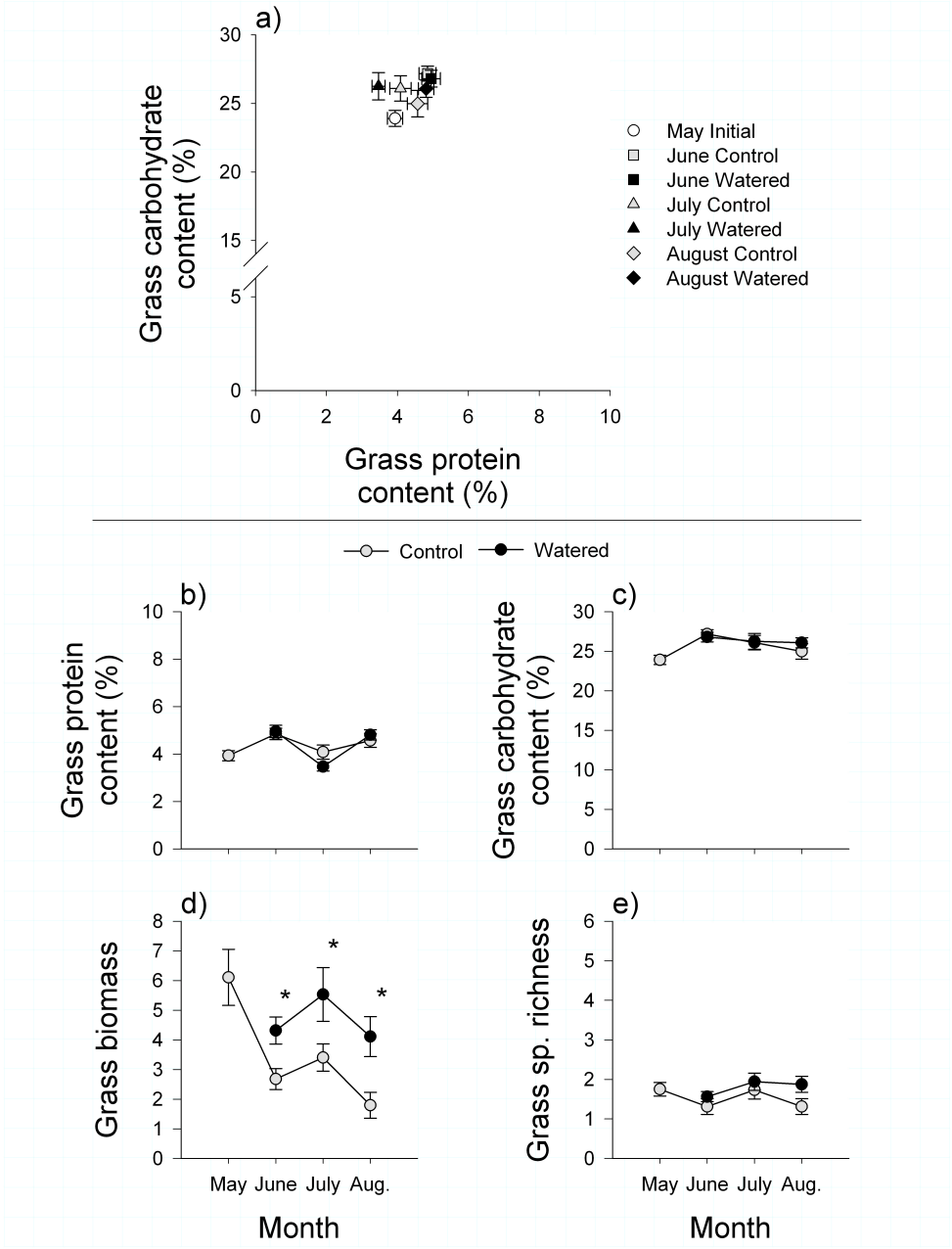

Fig. 3.1 Grass responses to water stress. (a) Protein and digestible carbohydrate biplot, (b) soluble protein content, (c) digestible carbohydrate content, (d) biomass (dry weight g/0.1m^2), and (e) species richness of grasses for control and watered plots across months (May-August). Mean and standard error bars are displayed.

Table 3.2 Results of MANOVA for protein and carbohydrate content of grasses and forbs. Analyses are shown for (a) grass and (b) forb both combined and with univariate analyses for protein and carbohydrate separately. Approximate F for the Roy's greatest root test statistic is given for multivariate comparisons, except for Treatment effects and all univariate comparisons, which has the exact F reported.

Source	Test	df	Prob>F
A) Protein and carbohydrate content of grass			
Site	6.12	3, 84	0.001*
Time	15.55	2, 84	<0.001*
Treatment	0.08	2, 83	0.922
Time*Treatment	2.28	2, 84	0.109
Protein content of grass			
Site	6.06	3, 84	0.001*
Time	15.54	2, 84	<0.001*
Treatment	0.02	2, 83	0.887
Time*Treatment	2.24	2, 84	0.112
Carbohydrate content of grass			
Site	2.62	3, 84	0.056
Time	1.74	2, 84	0.182
Treatment	0.13	2, 83	0.722
Time*Treatment	0.48	2, 84	0.618
B) Protein and carbohydrate content of forb			
Site	6.14	3, 84	<0.001*
Time	14.16	2, 84	<0.001*
Treatment	1.15	2, 83	0.322
Time*Treatment	3.45	2, 84	0.036*
Protein content of forb			
Site	1.38	3,84	0.254
Time	14.15	2, 84	<0.001*
Treatment	0.12	1, 84	0.727
Time*Treatment	1.73	2, 84	0.184
Carbohydrate content of forb			
Site	5.33	3,84	0.002*
Time	0.36	2, 84	0.699
Treatment	2.03	1, 84	0.158
Time*Treatment	1.34	2, 84	0.268

content varied significantly between sites, with marginally significant variation in carbohydrate content (Table 3.2a).

Grass biomass was consistently higher in watered plots (Fig. 3.1d; ANOVA, treatment: $F_{1,1}$=4.30, *P*=0.041, time*treatment $F_{2,2}$=0.21, *P*=0.814), differed across the four sites (ANOVA, site: $F_{3,3}$=3.66, *P*=0.015), and decreased from May to August (ANOVA, time: $F_{2,2}$=5.07, *P*=0.008). Grass species richness was unaffected by the watering treatment, (Fig. 3.1e; ANOVA, treatment: $F_{1,1}$=0.80, *P*=0.374, time*treatment: $F_{2,2}$=0.48, *P*=0.621) and remained constant throughout the growing season (ANOVA, time: $F_{2,2}$=2.07, *P*=0.132).

3.4.2 Forbs responses

Forb protein-carbohydrate content showed greater variation (Fig. 3.2a-c) than grasses. As the growing season progressed, forbs in watered plots developed more protein-biased macronutrient profiles than unwatered forbs (Fig 3.2a-b, Table 3.2b). In August the average protein:carbohydrate (p:c) ratio of watered plots was 1:3, compared to 1:3.6 in unwatered plots. Protein-carbohydrate content also differed between sites and months (Table 3.2). Despite the change in p:c ratio, univariate tests found that, independently, neither protein or carbohydrate content was affected by watering, nor was there a significant time-by-treatment interaction (Table 3.2b). Protein content, but not carbohydrate content, varied between months (Table 3.2b); protein content dropped from June to July ($F_{1,84}$=20.76, *P*<0.001) and then increased in August ($F_{1,84}$=21.36, *P*<0.001). Carbohydrate content varied between sites (Table 3.2b).

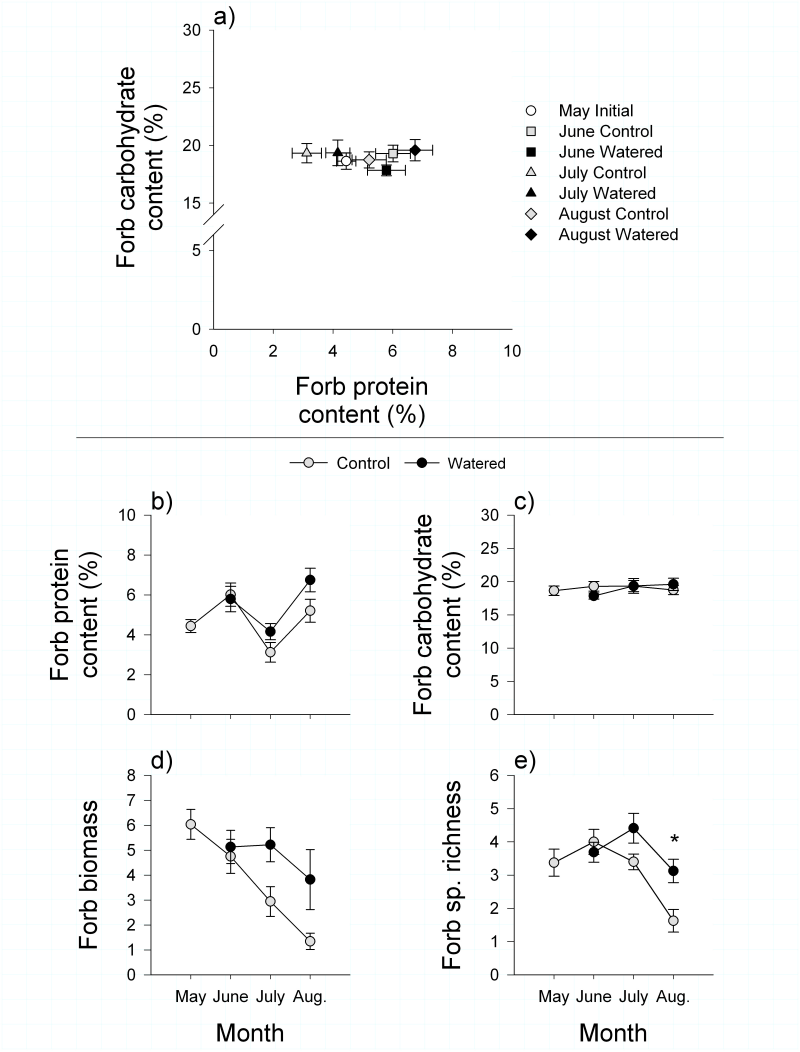

Fig. 3.2 Forb responses to water stress. (a) Protein and digestible carbohydrate biplot, (b) soluble protein content, (c) digestible carbohydrate content, (d) biomass (dry weight g/0.1m^2), and (e) species richness of forbs for control and watered plots across months (May-August). Mean and standard error bars are displayed. *Note:* When protein-carbohydrate content was analyzed using MANOVA (see Table 3.2b), a significant difference between the control and watered plots was detected for August (the grey and black diamonds, respectively).

Forb biomass was unaffected by the watering treatment (ANOVA, treatment: $F_{1,1}$=0.14, *P*=0.713, time*treatment: $F_{2,2}$=1.11, *P*=0.336) and decreased steadily over the course of the summer (Fig. 3.2d; ANOVA, time: $F_{2,2}$=5.62, *P*=0.005). The number of forbs species in any given plot decreased over the summer (Fig. 3.2e; ANOVA, time: $F_{2,2}$=12.09, *P*<0.001), but the rate of decline was significantly higher in unwatered plots (ANOVA, time*treatment: $F_{2,2}$=3.54, *P*=0.033).

3.4.3 Grasshopper responses

Total grasshopper density declined in all field plots during the growing season, but this decline was dramatically faster in unwatered plots (Fig. 3.3a, Table 3.3). By the end of the experiment, grasshopper abundance in control plots was almost three times lower than in watered plots (χ^2=20.27, df =1, *P*<0.001). Grasshopper species richness followed the same pattern (Table 3.3, Fig. 3.3b) with significant differences between treatments evident in August (χ^2=7.91, df =1, *P*=0.005). The number of grasshopper species in all plots decreased during the course of the experiment, but in control plots this occurred at a faster rate compared to watered plots (Table 3.3, Fig. 3.3b).

What happened when grasshoppers were examined as functional feeding groups? Grass-feeding grasshoppers responded positively to watering supplementation (Fig. 3.3c, d, Table 3.3). Between May and July the density of grass-feeding grasshoppers varied little. However, in August density in watered plots nearly doubled while the density in unwatered control plots decreased (χ^2=12.80, df =1, *P*<0.001). The species richness of grass-feeders also increased in watered plots, but decreased in control plots in late

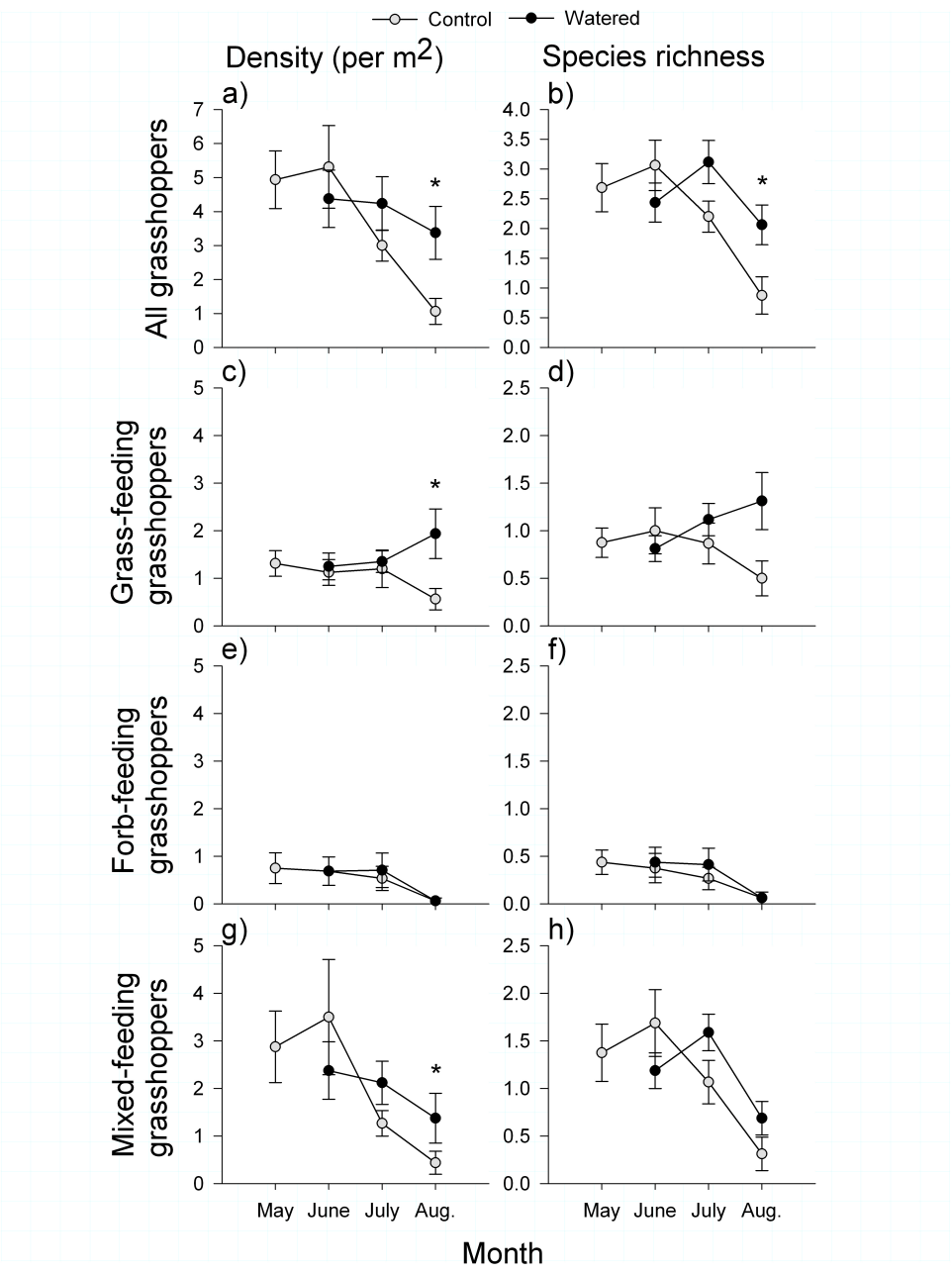

Fig. 3.3 Grasshopper responses to water-stressed plants. Grasshopper density and species richness (mean ± SE) are shown for control and watered plots across months (May-August) for all grasshopper species combined (a,b) as well as the three functional grasshopper feeding groups separately: (c,d) grass specialists, (e,f) forb specialists, and (g,h) mixed-feeding grasshoppers.

Table 3.3 The effects of time and treatment on density and species richness for all grasshoppers combined, as well as forb, grass, and mixed-feeding grasshoppers separately. Results are based on analysis with Generalized Linear Models (Poisson distribution).

		Density			Species richness		
Feeding group	Source	df	χ^2	P>χ^2	df	χ^2	P>χ^2
All grasshoppers	Block (Site)	3	60.97	<0.001*	3	13.30	0.004*
	Time	2	39.61	<0.001*	2	17.69	<0.001*
	Treatment	1	1.45	0.228	1	1.14	0.286
	Time*Treatment	2	19.95	<0.001*	2	8.90	0.012*
Grass-feeding	Block (Site)	3	29.82	<0.001*	3	16.99	0.001*
	Time	2	0.69	0.707	2	0.52	0.769
	Treatment	1	0.11	0.746	1	0.31	0.577
	Time*Treatment	2	7.70	0.021*	2	4.77	0.092
Forb-feeding	Block (Site)	3	33.28	<0.001*	3	16.59	0.001*
	Time	2	21.99	<0.001*	2	9.67	0.008*
	Treatment	1	0.00	1.000	1	0.08	0.781
	Time*Treatment	2	0.35	0.840	2	0.22	0.896
Mixed-feeding	Block (Site)	3	51.17	<0.001*	3	4.21	0.240
	Time	2	39.27	<0.001*	2	18.01	<0.001*
	Treatment	1	3.47	0.062	1	1.40	0.237
	Time*Treatment	2	14.65	0.001*	2	4.95	0.084

summer (Fig. 3.3d); this pattern was only marginally significant (Table 3.3). Forb-feeding grasshoppers, on the other hand, were unaffected by the watering treatment (Table 3.3, Fig. 3.3e-f). The abundance of mixed-feeding grasshoppers declined over the course of the growing season, but was higher in watered plots (Fig 3.3g-h, Table 3.3). From May to June mixed-feeder density did not change, although, control plots in June trended towards higher density (χ^2=3.47, df =1, *P*=0.063). Between June and July, densities in both control and water-treated plots declined. However, in July there were marginally more mixed-feeding grasshoppers in watered plots than control plots (χ^2=3.73, df =1, *P*=0.053), but in August this difference was significant (χ^2=8.15, df =1, *P*=0.004). Over the course of the summer the species richness of these mixed-feeding grasshoppers declined, with a marginally significant trend for more species in watered plots compared to control plots in later months (Fig. 3.3h, Table 3.3).

3.5 Discussion

Previous studies exploring the effects of water stress on plant nutritional quality have focused primarily on nitrogen (N), amino acids, and/or protein (White 1984, Franzke and Reinhold 2011). However, water stress influences multiple plant primary metabolites (Mattson and Haack 1987). Furthermore, because insect herbivore performance is determined by both the amounts and ratios of multiple nutrients, especially protein and digestible carbohydrates (Raubenheimer and Simpson 1999, Behmer 2009), working in a single nutritional dimension (e.g., N) fails to adequately capture how water-stress impacts plants as nutritional resources for insect herbivores.

My results indicate that water-stress affects the p:c ratio of forbs but not grasses. A key aspect of my approach was to also focus on grasshopper responses to water inputs, and generally I found more grasshoppers (particularly grass- and mixed-feeding grasshoppers) in water-supplemented plots. By combing plant and grasshopper responses to water stress, my study provides novel insights into how water stress can affect plant-insect herbivore interactions.

My results provide a quantification of the plant protein-carbohydrate landscape available to insect herbivores in a grassland ecosystem. I tracked protein and carbohydrate content of native grasses and forbs across a growing season, across different fields, and between watered and unwatered plots. Interestingly, grasses and forbs responded differently to the watering treatment but neither followed the prediction that protein and carbohydrate content would increase under water stress. Grasses displayed similar macronutrient profiles on both watered and ambient controlled plots and this occurred despite several months of the worst drought in recorded Texas history (Nielsen-Gammon 2011). Grass biomass increased in watered plots, possibly due to increased uptake of limiting nutrients (N and P) locked in the soil (Lambers et al. 2008), but the grasses maintained the same average foliar protein and carbohydrate content. Previous water stress studies have found mixed effects of drought on protein content in grasses. Among C3 grasses water stress has been reported to cause both increases and decreases in protein (Franzke and Reinhold 2011, Walter et al. 2012). In C4 grasses, Barnett and Naylor (1966) found that soluble protein decreased with water stress. These three studies utilized greenhouse-grown plants. In contrast, my study utilized

established, drought acclimated, perennial C4 bunchgrasses, which use water more efficiently than C3 grasses (Ghannoum 2009). That my grasses were drought hardened in the field, not young greenhouse reared plants, may explain the lack of a macronutrient shift in my study.

In forbs, protein and carbohydrate concentration did not shift in a significant fashion, but I did observe a significant shift in the p:c ratio. Specifically, forbs in watered plots became more protein biased over the course of the growing season. Surprisingly, it took weeks of continuous drought to observe a shift in forb p:c ratios (Fig. 3.2a). This resilience could be due to a number of drought resistance traits such as modification of root structure, osmotic adjustments, reduced stomatal conductance, increased transpiration efficiency, and high temperature tolerance (Ludlow and Muchow 1990). The eventual increases in protein content in water-supplemented forbs may reflect better uptake of soil N, leading to protein synthesis, which could be invested in growth and reproductive structures (Lambers et al. 2008).

Contrary to my predictions total grasshopper density and species richness increased on watered plots. Water supplementation established patches with more grass biomass, great forb diversity, and forbs with higher p:c ratios. Due to the scale of the experiment ($1m^2$ plots) density changes were likely due to grasshoppers aggregating in watered plots, not changes in survival or reproduction. During drought, insect herbivore populations may become more patchily distributed on surviving vegetation, for example in mesic habitat. In some instances, high densities of insect herbivores on remaining vegetation patches (during a drought) may give an impression that a given species has

undergone an ‘outbreak’(Mattson and Haack 1987). In the case of orthoptera species that exhibit phase polyphenism (none occur at my site) drought induced patchiness can lead to outbreaks brought on by crowding (Despland et al. 2000).

However, treating all grasshopper species as a single taxonomic group (at the family level: Acrididae) may fail to account for potentially important biological differences associated with functional feeding groups. For example, previous studies have shown that herbivores in different feeding guilds (e.g., leaf chewers, phloem feeders, etc.) respond differently to water-stressed plants (Schowalter et al. 1999, Huberty and Denno 2004). My findings show that even within a family of physiologically similar leaf-chewing insects (Acrididae), response to a drought-stressed plant community differed. I suspect that these differences reflect each functional feeding group’s host plant response. Previous work has shown that different grasshopper functional groups also show differential responses to weather, fire, and bison grazing (Jonas and Joern 2007).

Grasshoppers specializing on grasses showed a strong response to watering in the final month of the experiment; more of these grasshoppers were counted in watered plots relative to ambient plots. Grass-feeding grasshopper may have been responding to grass biomass, as numbers did track with changes in grass biomass; responses to other grass traits were not observed. With respect to forb-feeding grasshoppers, density and species richness were unaffected by the watering treatment despite water treatment effects on forb p:c ratio and diversity. Although forb diversity increased with water, forb-feeding species are functionally different from other generalist grasshoppers in that they

specialize on only a few, related species of plants that share common similar defensive chemistry (Traxler and Joern 1999, Pfadt 2002). That forb-feeding grasshoppers did not track changes in forb p:c ratio suggests that subtle shifts in plant nutrient content are less important than secondary plant compounds, which identify plants as suitable food plants (Bernays and Chapman 1994). Finally, the response of mixed-feeder grasshoppers is likely a result of their diet-mixing feeding ecology. Mixed-feeder abundance declined at a slower rate as the drought progressed on watered plots, and this change was associated with higher grass biomass, a more protein-biased forb macronutrient profile and higher forb species richness. Although mixed-feeders utilize both grass and forbs, most of these species mainly feed on forbs (Joern 1985). Mixed-feeders tightly regulate macronutrient intake via diet mixing (Behmer and Joern 2008), so a higher forb species richness would allow generalist grasshoppers greater flexibility with respect to choices related to diet mixing. This can lead to better nutrient intake and dilutes any one plant's allelochemical defenses (Hagele and Rowell-Rahier 1999, Behmer et al. 2002, Singer et al. 2002).

My approach reveals novel insights concerning how water inputs can affect plant-herbivore interactions, but how can these results be extended more broadly? A key challenge for ecologists and modelers of climate change is the need to understand and incorporate biotic interactions in models of future climactic conditions (Van der Putten et al. 2010). My findings could be used to integrate multiple theoretical frameworks in nutritional ecology (Raubenheimer et al. 2009, Kearney et al. 2010, Simpson et al. 2010) into models of the effects of global change. Simpson et al. (2010) proposes combining advancements in agent-based models, state-space models of nutrition, and multi-scaling

modeling of landscape ecology. The result could be used to predict how individual herbivores will forage in a variable landscape of plant quality and could be scaled up to investigate how changes in plant quality affect population, community, and ecosystem dynamics. The ability to remotely sense for changes in plant nutrient content (Foley et al. 1998, Zengeya et al. 2013) and other plant characteristics such as water content (Ullah et al. 2012b), biomass (Ullah et al. 2012a), and possibly even plant diversity (Gould 2000) allows for model validation and real world application at landscape and regional scales. My study provides valuable quantitative data for parameterizing models concerned with how different plants (C4 grasses and C3 forbs) and insect herbivores (grass-specialists, forb-specialists, and mixed-feeding grasshoppers) respond to a key environmental variable: water availability. My study also demonstrates that caution must be taken to not over simplify the biology of the study organisms. Future model development needs to integrate the variable effects of water stress on the biology of plant and animal functional groups.

To model the response of insect herbivores in diverse plant communities under projected future climatic conditions, further work needs to quantify how various plant functional groups responds in ecosystems of interest. Field studies may give more realistic results than greenhouse trials. In addition to the plant variables measured in this study, future work should take into account other plant traits that concern herbivores such as defensive chemistry, grass silica content, water content, toughness, and variation in macronutrient content between plant species to obtain the entire range of the nutrient landscape available to a foraging herbivore. To more completely understand how

drought responses in plants affect these herbivore-relevant traits, detailed plant physiological measures such as water potential, relative water content, and photosynthetic rate need to be monitored. Only by collecting comprehensive data on how plants and insect herbivores respond to water stress in the field can we construct and parameterize robust models projecting interactions under future climactic conditions.

CHAPTER IV

MACRONUTRIENT REGULATION IN COEXISTING GENERALIST HERBIVORES

4.1 Overview

The fundamental hypothesis in nutritional ecology is that organisms selectively feed to best match their nutritional requirements. Active nutrition regulation, particularly with respect to protein and digestible carbohydrates, has now been shown in multiple species. Comparative studies of nutrient regulation could lead to a transformative understanding of why species differ in foraging and consumption behavior. Furthermore, it has been suggested that coexisting species that share common food resources might occupy unique nutritional niches as a mechanism to facilitate coexistence. Understanding how communities of organisms vary in terms of their nutrient regulation strategies may shed new light on factors enabling coexistence and structuring communities. My study identified self-selected protein-carbohydrate intake for a suite of coexisting grasshopper species. I investigated the intake results in the context of relatedness (taxonomy), host plant range, and body size to better understand factors that might determine a species' protein-carbohydrate preferences. I collected grasshoppers from the field as nymphs, reared each species in the laboratory, and subjected them to artificial diet experiments to determine their self-selected protein-carbohydrate intake. I documented host plant use for each species via microscopic analysis of gut content. My laboratory feeding studies revealed that all species actively regulated for protein-

carbohydrate intake. Self-selected protein-carbohydrate intake across the entire community was more carbohydrate-biased than previous studies. Gut content analysis of 11 species allowed me to assign species to functional feeding groups and showed that many species were highly polyphagous with broad overlap in host plant use. The ratio of protein:carbohydrate regulated for differed between species, and corresponded to differences in diet. Differences in nutrient requirements among related coexisting species with overlapping diet could be related to taxonomic and diet differences. In the case of mixed-feeding Melanoplinae, differences could reflect nutrient niche partitioning. I discuss my findings in the context of latitudinal gradients, body size, relative abundance, niche partitioning, and adaptive physiology.

4.2 Introduction

Nutrient regulation is a basic aspect of organismal biology, allowing optimization of growth and reproduction. The fundamental hypothesis in nutritional ecology is that organisms have evolved adaptations to the nutritional content and availability of their respective foods (Raubenheimer et al. 2009). A growing body of literature is beginning to address how nutritional requirements vary across individuals, populations, and species (Jaenike and Markow 2003, Raubenheimer and Simpson 2003, Lee et al. 2006, Behmer and Joern 2008, Parsons 2011). Many organisms actively regulate for a particular blend of nutrients in food (Richter et al. 1938, Waldbauer and Friedman 1991, Simpson et al. 2006, Behmer 2009, Simpson and Raubenheimer 2012). The blend of nutrients that an organism actively regulates for should include the nutrient requirements of the organism

but there may be other selective pressures on nutrient regulation (Sterner and Elser 2002). Comparative studies of nutrient regulation could lead to a transformative understanding of why species differ in foraging and consumption behavior. Moreover, differences in nutrient regulation across species and populations have major ecological implications and could lead to better predictive power in community ecology (Huey and Pianka 1981, Beckerman et al. 2010, Simpson et al. 2010, Kaspari et al. 2012).

A significant hurdle for a comparative study is that nutritional requirements and regulatory processes are complex, multidimensional, and difficult to define. However, recent advances in nutritional ecology using insect herbivores have demonstrated that digestible levels of protein and carbohydrates dominate the food selection choices of animals (Simpson and Raubenheimer 2012). To explicitly define regulation of these macronutrients, we can consider an organism within a nutrient space where axes are functionally relevant macronutrients (Simpson and Raubenheimer 2012). An animal's nutrient intake target (IT) represents the amount of nutrients an animal actively regulates for by adjusting the amount of an individual food eaten, eating different foods, or combining both. Investigators using this method (termed the 'Geometric framework') have demonstrated macronutrient intake targets in many taxa including humans, other mammals, birds, fish, slime molds, and insects (Raubenheimer and Simpson 1997, Simpson and Raubenheimer 2001a, Simpson and Batley 2003, Behmer 2009, Felton et al. 2009, Dussutour et al. 2010).

The geometric framework can be used as a powerful standardized tool to compare nutrient regulation across groups of interest. To date the most studied group is

insect herbivores (Behmer 2009), and in particular Orthoptera, which make an ideal model system. More species of Orthoptera have been studied using this technique than any other group however only 12 species have had their self-selected intake target determined (Chambers et al. 1995, Simpson et al. 2002, Clissold et al. 2006, Behmer and Joern 2008, Fielding and Defoliart 2008, Boswell 2009, Goeriz Pearson et al. 2011, Parsons 2011, Cease et al. 2012). Significant variation in the macronutrient requirements of different Orthoptera species has been found where comparisons were made (Simpson and Raubenheimer 1993, Behmer and Joern 2008). Nutritional ecologists hypothesize that these differences are the result of adaptive evolution (Raubenheimer and Simpson 1997, 1999, Simpson and Raubenheimer 1999, Raubenheimer and Simpson 2003) but many comparisons emphasized distantly related taxa or only compared a pair of species. Therefore, to adequately address the biologically relevant sources of variation in nutrient regulation, comparative studies need to be done incorporating more species with populations sampled from the field. Furthermore comparisons need to be made with how the organisms feed in nature if predictions based on nutrient requirements are to be extended to community dynamics.

My objectives were to 1) determine among a suite of insect herbivores whether all species regulate for specific ITs, 2) establish if species differ in their nutrient requirements, and 3) investigate whether any patterns exist among nutrient requirements with respect to taxonomy, diet, or body size. For a suite of coexisting grasshopper species from a Central Texas grassland, I experimentally determined the self-selected protein:carbohydrate intake of each species and described the diet of field-collected

individuals through gut content analysis. Based on previous work (Chambers et al. 1995, Simpson et al. 2002, Behmer and Joern 2008, Behmer 2009, Clissold et al. 2009) I hypothesized that grasshopper species would not feed randomly but rather regulate for specific intake targets and grasshopper ITs would occupy a similar range near 1:1 protein:carbohydrate. I also hypothesized that intake targets would be similar for closely related species given their common ancestry and would differ across different higher-level taxa (subfamilies). I investigated the hypothesis that ITs would be related to grasshopper diet and that ITs could reflect the nutritional content of food (Joern & Mole 2005; Behmer 2009). For example, since grasses are more carbohydrate-biased than forbs I hypothesized that the higher the proportion of grass in a species diet, the more carbohydrate-biased the IT should be. Finally I tested whether protein:carbohydrate intake varies with body size.

4.3 Methods

4.3.1 Study system

This study was conducted at the Balcones Canyonlands National Wildlife Refuge (BCNWR) northwest of Austin, Texas. The area hosts a diverse grasshopper community with at least 56 different species of Acrididae. The refuge covers parts of Burnet, Williamson and Travis Counties. The geology of the study site is characteristic of the Edwards plateau with limestone hills and shallow rocky soils. All grasshoppers were collected by sweep netting areas of mixed-grass prairie and oak (*Quercus* sp.) savannah. Eleven grasshopper species were included in this study (Table 4.1). These species were

chosen to because they were abundant enough to facilitate the collection for laboratory diet experiments, represented a range of grasshopper subfamilies (Gomphocerinae, Melanoplinae, and Oedipodinae), represented a range of diet groupings (grass-, forb-, and mixed-feeders), and fed on the diet with >90% survival and normal development time (<15 days for last nymphal instar). All Melanoplinae and Oedipodinae species

Table 4.1 MANCOVA results for diet pairings among grasshopper species. If each species is actively regulating protein-carbohydrate intake than no significant difference in protein-carbohydrate consumption should be observed between (Joern and Mole 2005, Behmer 2009) treatments. Initial wet mass was used as a covariate.

Taxa	Df	Exact F	Prob>F
Gomphocerinae			
Ageneotettix deorum (Scudder)	2, 31	0.16	0.853
Melanoplinae			
Hesperotettix speciosus (Scudder)	2, 16	0.07	0.937
Melanoplus differentialis (Thomas)	2, 22	6.94	0.005*
Melanoplus discolor (Scudder)	2, 34	2.41	0.105
Melanoplus femurrubrum (De Geer)	2, 23	2.26	0.127
Melanoplus flabellatus (Scudder)	2, 28	1.23	0.309
Melanoplus packardii Scudder	2, 17	0.75	0.486
Melanoplus ponderosus (Scudder)	2, 17	2.55	0.109
Phaedrotettix concinnus (Scudder)	2, 25	0.77	0.475
Oedipodinae			
Hadrotettix trifasciatus (Say)	2, 22	0.95	0.401
Spharagemon equale (Say)	2, 24	1.27	0.298

sampled fed on the artificial diet readily. In the Gomphocerinae, *Mermiria bivitatta* (Serville) and *Boopedon gracile* Rehn would not feed on the artificial diet and died, however *Ageneotettix deorum* (Scudder) did and was included in the study.

4.3.2 Nutrient intake target analysis

To define nutrient ITs I collected grasshopper nymphs from the BCNWR during May and June of 2011-2013. I sorted the field-collected grasshopper nymphs to species and fed them a mixture of organic wheat seedlings and romaine lettuce. Grasshoppers were kept in a laboratory rearing room for a minimum of 48 hrs after collection so that any individuals injured during collection would die. After 48hrs I removed all last instar grasshoppers and monitored the cages several times a day for any individuals molting into the final nymphal instar. The insects in the final nymphal stadium, the 5^{th} for all species with the exception of *Melanoplus differentialis* (6^{th}) and *Hadrotettix trifasciatus* (4^{th}), were used because their large body size corresponds with larger amounts of food eaten, yet, since they are not adults little feeding is dedicated to reproductive development. Only somatic growth was expected. The choice experiment used artificial diets with variable protein: carbohydrate, allowing the grasshopper to self-select its preferred macronutrient intake. Upon molting into the final nymphal stadium, I recorded the mass of individual nymphs and placed them into marked 18.9 x 13.3 x 9.6 cm plastic arenas. All masses were recorded to the nearest 0.01mg. Grasshoppers in the experimental arenas were kept at 38°C during daytime and 27°C at night with a 14:10 hr light:dark photoregime. Each arena had a wire mesh perch, a water-filled small sealed

plastic cup with a cotton wick for drinking, and a pairing of two dried granular artificial diets.

Diets were prepared using the methodology of Dadd (1961) and Simpson and Abisgold (1985) with definite amounts of digestible protein and carbohydrates to calculate the % protein: % carbohydrate (henceforth p:c) ratio which each species regulated for. The IT for each species can be determined by allowing insects to feed between two nutritionally suboptimal but complementary foods. I used diets with p:c ratios of 7:35, 35:7 and 28:14. These ratios bracket the known variation in grasshopper ITs around 1:1 (Behmer 2009). Experimental treatments in this choice experiment used the combinations of 7:35 vs. 35:7 and 7:35 vs. 28:14. Two treatments are necessary to verify that ITs are not simply the result of grasshoppers eating equally from the two available foods. I allowed the diet dishes to equilibrate with the ambient humidity in the laboratory for 24-48hr and then weighed them prior to placing them in experimental arenas. Diets were changed after 6 days and weighed after equilibrating in the lab for 24hrs. Arenas were checked multiple times a day, and upon molting into the adult stage, grasshoppers were weighed (wet mass) and frozen. I recorded final diet weights after equilibration in the lab.

For the nutrient intake analysis the amount of diet consumed from each dish and the protein and carbohydrate content of each diet were used to calculate the total amounts of protein and carbohydrate consumed. Data from day 0-6 of the final nymphal instar was used for all calculations. I first analyzed consumption within each species to determine whether species were actively feeding for a specific p:c intake target, or

feeding randomly. I compared protein and carbohydrate consumption against sex and diet treatment using MANCOVA with initial wet mass as a covariate. Univariate tests (protein and carbohydrate consumption separately) were conducted using ANCOVA with initial wet mass as a covariate. After establishing that each species defended an intake target, I combined treatments within a species and tested for differences between species. I compared interspecific differences in macronutrient consumption corrected for body size (protein eaten after 6 days/initial wet mass, carbohydrates eaten after 6 days/initial wet mass) using MANOVA. I report the Wilk's Lambda test statistic. All analyses on nutrient intake were conducted in JMP 10 (JMP 1998-2007).

4.3.3 Grasshopper gut content analysis

Grasshopper diets were determined by identifying plant fragments in gut contents using a compound microscope (Isely and Alexander 1949, Joern 1979a, Sword and Chapman 1994, Sword and Dopman 1999). On June 2, 2011, I collected grasshoppers on the BCNWR by sweep netting areas of mixed-grass prairie and oak savannah. I preserved specimens in 70% ethanol and later dissected crops from late instar nymphs of 11 grasshopper species (n=7-18) and wet mounted the contents on slides. I collected reference specimens of 57 plant species (16 families) from BCNWR grasslands between May to July of 2011. Plant specimens were identified and vouchers deposited in the S.M. Tracy Herbarium at Texas A&M University (http://essm.tamu.edu/facilities/sm-tracy-herbarium/). I removed small pieces of leaves from each specimen, microwaved them for 30 seconds in water, and scraped away a section of the leaf epidermis using a

razorblade. Plant scrapings were wet mounted on slides. Slides of both grasshopper crop contents and reference plant specimens were analyzed and photographed using a Motic® BA410 microscope at 40-400x. Features such as trichomes, cell shapes, vascular tissue patterns, and leaf edge morphology were used to identify host plants by comparison with the photos from the reference specimens. I visually estimated proportion of each diet item (plant or arthropod morphospecies) in each gut and summed totals for each grasshopper species. Plant taxa found in the gut were combined at the level of the plant family. This was done because 1) while some plant taxa were identifiable to species or genus, others could only be identified to family or less, and 2) it is well established that insect herbivores are able to select and feed on related plants due to the often similar phytochemistry (Eastop 1979, Hille Ris Lambers 1979, Bernays and Chapman 1994, Schoonhoven et al. 1998).

Niche overlap in terms of grasshopper diet was analyzed by comparing the Pianka index (Pianka 1973):

$$O_{jk} = O_{kj} = \Sigma\, p_{ij}p_{ik} / \sqrt{\Sigma\, pij^2\, p_{ik}{}^2}$$

In this index O_{jk} and O_{kj} represent the overlap between a species pair, with values ranging from 0 (no overlap) to 1 (complete overlap), and p_{ij} and p_{ik} represent the proportions of the i^{th} resource used by the j^{th} and k^{th} species, respectively. Niche overlap indices and analysis were calculated using EcoSim Professional (Entsminger 2012). Pianka's index was calculated using the frequency of each diet category consumed by the entire population for each species. I tested whether the observed mean trophic niche overlap between species differed significantly from a null model of expected niche

overlap when resources were randomly consumed. The observed diet content matrix was reshuffled 1000 times to generate a distribution of random expectations for niche overlap. This simulated dataset was created with the following stipulations: 1) there was an equal probability that resources could be consumed; 2) niche breadth was retained, meaning the degree of specialization for each species was preserved; and 3) zero states were reshuffled so that species could use resources in the null communities that were not consumed in the observed data (Gotelli and Graves 1996). The null hypothesis (the observed niche overlap is no different than expected under random consumption of resources) was rejected if the observed overlap was < 2.5% or > 97.5% of the expected overlap values.

4.3.4 Sources of nutrient intake target differences

Currently this system lacks enough replication across groups of interest (diet groupings, higher order taxa) as well as a resolved phylogenetic tree including all sampled grasshopper taxa (Chapco and Litzenberger 2002, Contreras and Chapco 2006, Fries et al. 2007, Song 2010) to perform phylogenetically controlled analyses (Garland et al. 2005). However, this is the largest set of coexisting species with variable diet that have thus far been sampled for self-selected macronutrient ITs and so I can make contrasts between groups of interest. I focused on differences in IT ratio rather than absolute amounts of protein and carbohydrate consumed because 1) the amount consumed is strongly related to body size and 2) ratio disparities require selecting different host tissues which is more biologically relevant. Species with similar intake

ratios but dissimilar absolute amounts of protein and carbohydrate required can theoretically feed on the same food but consume different amounts. I calculated the p:c ratio selected by each individual grasshopper and compared species means using ANOVA with log transformed ratios. Ratios were log transformed as this more accurately reflects linear differences between p:c ratios; *e.g.*, the values of 1:2 and 2:1 are -0.301 and 0.301 which are equidistant to a balanced 1:1 ratio (log(1/1) = 0), rather than 0.5 and 2. I constructed orthogonal contrasts between species with the goal of testing for IT differences across subfamilies and diet of each species (based on the gut content analysis). Contrasts are listed in Table 4.2. Comparisons among the six mixed-feeding Melanoplinae were structured using the results of a clustering analysis (hierarchical cluster, Ward's minimum variance method) of the individual diet items (Appendix Table A.3) consumed by each species. The ANOVA, orthogonal contrasts, and cluster analysis were all performed using JMP 10 (JMP 1998-2007). I also tested whether IT ratio (log transformed) was related to body size using a general linear model (GLM) including species, initial wet mass (at the beginning of the final nymphal instar), and a species×initial wet mass interaction as predictor variables. I conducted the GLM with all species as well as separately with only mixed-feeding grasshoppers to eliminate any effects of species with specialized diet. The GLM was conducted using JMP 10 (JMP 1998-2007).

4.4 Results

4.4.1 Nutrient intake target

All 11 species analyzed actively regulated their feeding rather than feeding randomly from the artificial diet dishes. All grasshopper species, with the exception of *Melanoplus differentialis*, fed selectively on the two artificial diet treatments to reach the same p:c IT (Table 4.1). *Melanoplus differentialis* subjected to the diet pairing with more protein (7:35 vs. 35:7) consumed more protein (ANCOVA Protein: $F_{1,23}$=6.03 P=0.022*; Carbohydrate: $F_{1,23}$=0.57 P=0.459). Still, this intake point was close to the 7:35 vs. 28:14 diet treatment IT, and statistically different from a random intake. Nutrient intake experiments using other populations of *M. differentialis* in Texas with the same diet treatments found that the species did defend an IT (Le Gall in prep) and so there may be a Type II error in my result for this species. In all species the effect of sex was non-significant and removed from the model.

The coexisting species used in this study regulated for macronutrients differently. I found significant differences existed between the absolute amounts of protein and carbohydrate regulated for by the 11 grasshopper species tested (Fig. 4.1, Appendix Table A.2, MANOVA: species Wilk's Lambda $F_{20,570}$=12.45, P <0.001*). When I compared ratio alone, without the influence of absolute amounts of protein and carbohydrate consumed, again I found significant differences between the species (Fig. 4.2, ANOVA $F_{10, 282}$=9.87, P <0.001*). Protein:carbohydrate intake ratios were generally carbohydrate biased and ranged from nearly balanced (0.95:1 in *P. concinnus*) to almost double the amount of carbohydrate per unit protein (0.48:1 in *H. speciosus*).

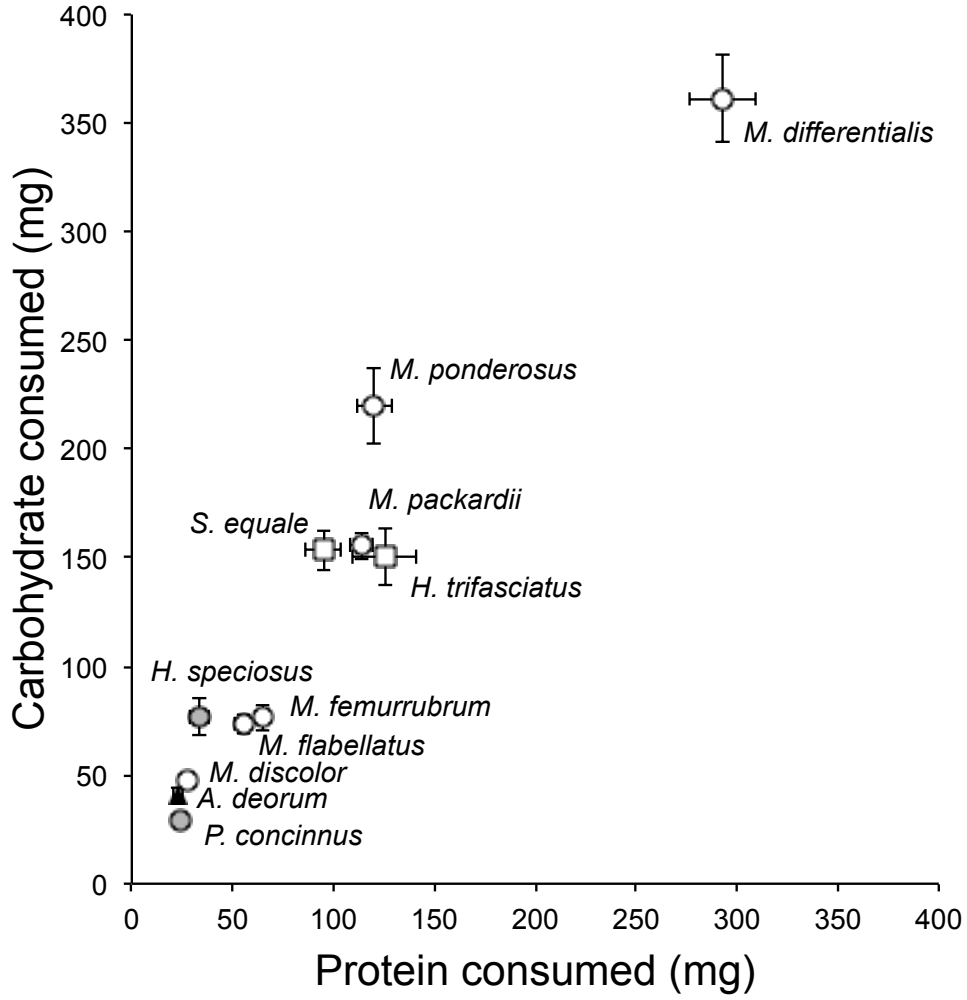

Fig. 4.1 Protein and carbohydrate consumption for 11 grasshopper species from Central Texas. Intake targets shown reflect the amount of macronutrients consumed in artificial diet choice experiments during the first six days of the final nymphal instar. Marker shape represents the grasshopper subfamily: Gomphocerinae (triangle), Oedipodinae (square), and Melanoplinae (circle). Fill color represents the functional feeding group as determined by gut content analysis: grass-feeder (black), forb-feeder (gray), and mixed-feeder (white).

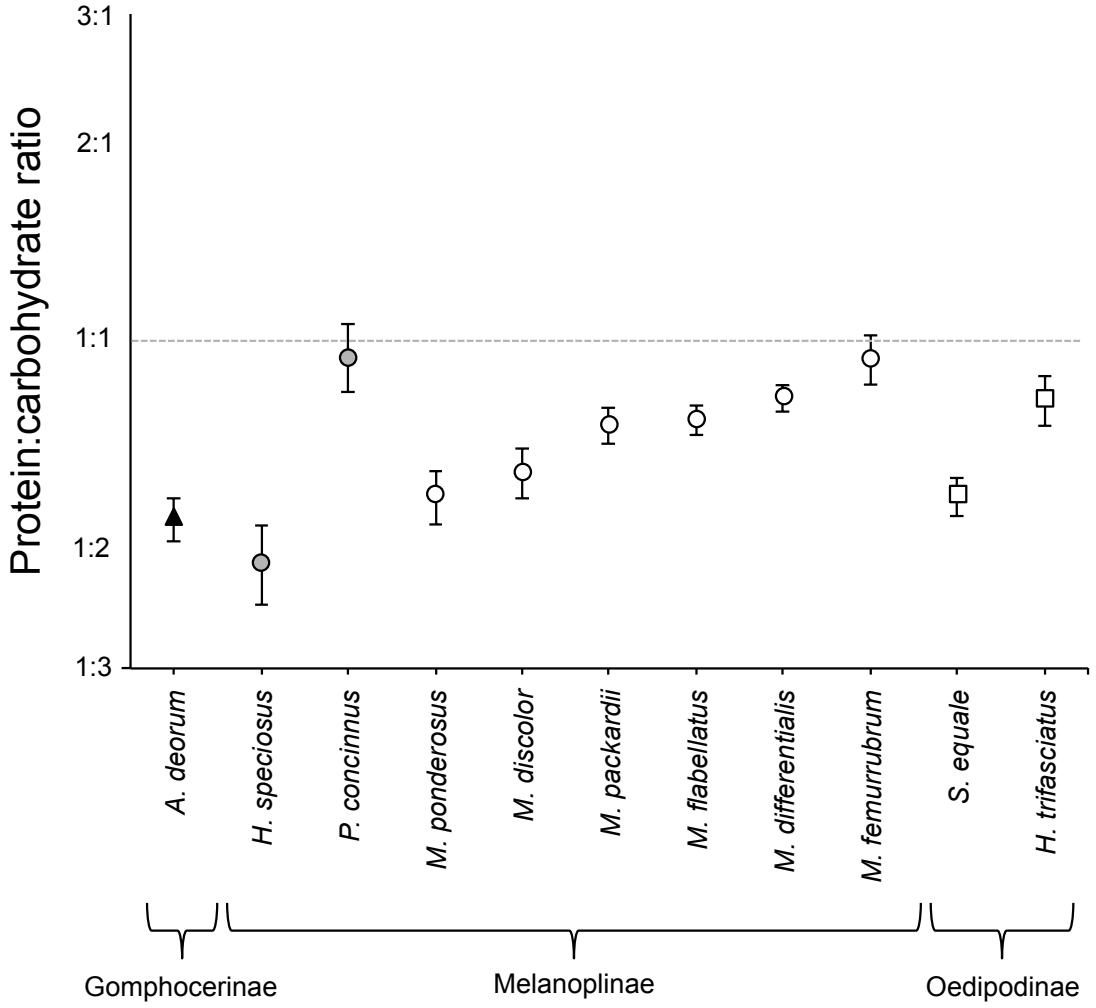

Fig. 4.2 Protein:carbohydrate ratio consumed for 11 grasshopper species from Central Texas. Protein:carbohydrate ratio is presented on a log scale to more accurately reflect the distance between ratios around a balanced 1:1 intake. Species are organized by subfamily and functional feeding group as determined by the gut content analysis. Marker shape represents the grasshopper subfamily: Gomphocerinae (triangle), Oedipodinae (square), and Melanoplinae (circle). Fill color represents the functional feeding group: grass-feeder (black), forb-feeder (gray), and mixed-feeder (white).

4.4.2 Grasshopper gut content

Grasshopper functional feeding groups were determined based on gut content with 1 grassfeeder (*A*. *deorum*), 2 grass feeders (*H*. *speciosus* and *P*. *concinnus*), and the remaining melanoplines and oedipodines being mixed feeding (Fig. 4.3, Appendix Table A.3). Most of the grasshopper species sampled were highly polyphagous while f species had relatively restricted diets (*A. deorum, H. speciosus*, *P. concinnus*, and *M. differentialis*). The most polyphagous feeders included widely distributed western species such as the melanopline *Melanoplus packardii* (9 plant families) and the oedipodine *Hadrotettix trifasciatus* (8 plant families) with the remaining mixed feeders feeding on 4-7 different plant families. Mixed feeders generally incorporated less than 15% grass in their diet with the exception of *M*. *differentialis* (54%). Arthropod parts were found in the crops of three species (*A*. *deorum*, *P*. *concinnus*, *H*. *trifasciatus*).

Diets of the 11 grasshopper species examined displayed a high degree of resource utilization overlap (Fig. 4.3, Appendix Table A.3). The comparison of the Pianka indices (Appendix Table A.4) of the sampled and simulated communities revealed that niche overlap was significantly greater than the expected level of overlap (observed index = 0.450, mean of simulated indices = 0.260, $P < 0.001$). This means that, in general, species were sharing trophic niches. On average, 90% of a grasshopper species diet was composed of diet categories, *i.e.*, plant morphospecies (Appendix Table A.3), which were shared by another grasshopper species. The only plant families that were exclusively fed on by one grasshopper species sampled were Apocynaceae (*H*.

trifasciatus), Euphorbiaceae (*M. ponderosus*), Plantaginaceae (*M. packardii*), and Solanacaeae (*M. packardii*).

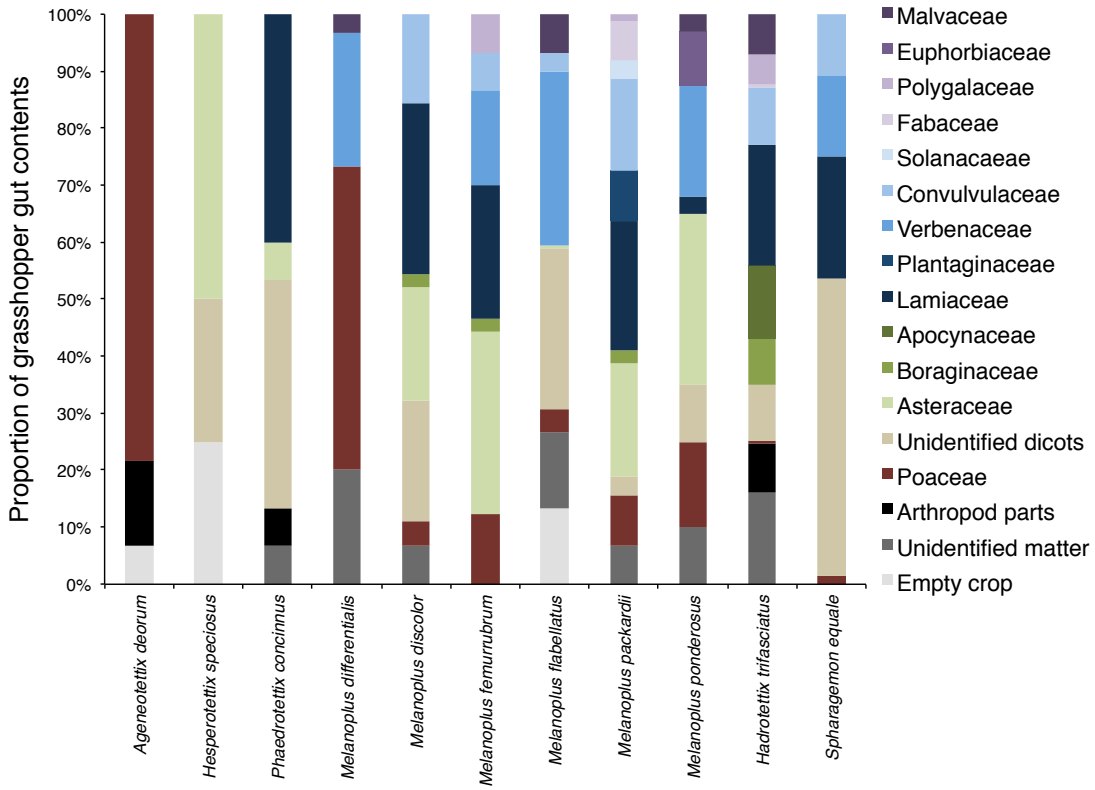

Fig. 4.3 Grasshopper diets as determined by crop content analysis. Each food category is represented by frequency at which it occurred among individual grasshopper crops from each sampled species pool. Sample sizes per species (n=7-18) are given in Appendix Table A.3. Dicots with no distinguishing feature, mainly no trichomes, are combined (Unidentified dicots). Based on my reference plant library this could potentially include plants within the Asteraceae, Euphorbiaceae, Fabaceae, Gentianaceae, and Rubiaceae.

Table 4.2 Comparison of protein:carbohydrate intake ratios. Comparisons were made using specified orthogonal contrasts designed using the subfamily and diet of each species. Contrasts within the mixed-feeding Melanoplinae (e-i) were structured using the results of a clustering analysis (Fig. 4.4) of the individual diet items consumed by each species (Appendix Table A.3).

Contrast	DF	F	Prob>F
a) *A. deorum* vs. non-grass specialists	1,282	16	<0.001*
Non-grass specialists			
b) Oedipodinae vs. Melanoplinae	1,282	0.12	0.73
Oedipodinae			
c) *H. trifasciatus* vs. *S. equale*	1,282	8.57	0.004*
Melanoplinae			
Forb-specialist Melanoplinae			
d) *H. speciosus* vs. *P. concinnus*	1,282	39.93	<0.001*
Mixed-feeder Melanoplinae			
e) *M. differentialis*/*M. flabellatus*/*M. ponderosus* vs. *M. discolor*/*M. packardii*/*M. femurrubrum*	1,282	0.513	0.474
f) *M. differentialis* vs. *M. flabellatus*/*M. ponderosus*	1,282	5.88	0.016*
g) *M. flabellatus* vs. *M. ponderosus*	1,282	7.404	0.007*
h) *M. femurrubrum* vs. *M. discolor*/*M. packardii*	1,282	9.6	0.002*
i) *M. discolor* vs. *M. packardii*	1,282	4.28	0.040*

4.4.3 Sources of nutrient intake target differences

The three subfamilies included in this study had high degrees of overlap in terms of IT ratio regulated for (Fig. 4.2), however the gomphocerine sampled tended to have a more carbohydrate-biased IT (*A. deorum,* 0.55:1) (Table 4.2a). Melanoplinae and Oedipodinae overlapped completely and were not significantly different (Table 4.2b). When species are segregated based on diet as determined by the gut content analysis there are 1 grass-feeder, 2 forb-feeders, and 8 mixed-feeders (Fig. 4.3). The one grass-feeder, also being the single gomphocerine has the same relationship described above.

The forb-feeders *H. speciosus* (0.48 :1) and *P. concinnus* (0.95:1) bracket the measured IT ratio variation (Fig. 4.2) and are significantly different from on another. Mixed-feeders spanned a wide range from very carbohydrate-biased (*M. ponderosus*, 0.59:1) to nearly balanced (*M. femurrubrum*, 0.94:1). Among the two mixed-feeding oedipodines, *S. equale* had a significantly more carbohydrate biased IT than *H. trifasciatus* (Table 4.2c). Among mixed-feeding Melanoplinae the cluster analysis separated species based on diet similarity (Fig. 4.4). There was no significant difference in ITs between the two biggest diet groups (as determined by the diet clustering analysis) (Table 4.2e). The two groups have the most dissimilar diets among the mixed-feeding Melanoplinae but they are interspersed in terms of their protein:carbohydrate intake (Fig 4.2). However, grasshopper species within these two clusters, species which have more similar diets to one another, all had significantly different IT ratios (Table 4.2f-i). Body size showed no relationship with IT ratio (Fig 4.5) when compared across all species (GLM; species: $F_{10,271}$=3.62, P <0.001*, initial wet mass: $F_{1,271}$=0.51, P =0.477, species×initial wet

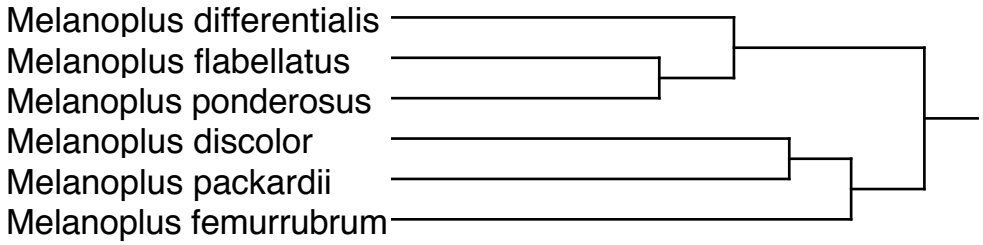

Fig. 4.4 Hierarchical cluster dendrogram (Ward's minimum variance method) relating diet similarity among mixed-feeding melanopline grasshoppers. The analysis compared the frequency of individual diet items consumed by each species (Appendix Table A.3).

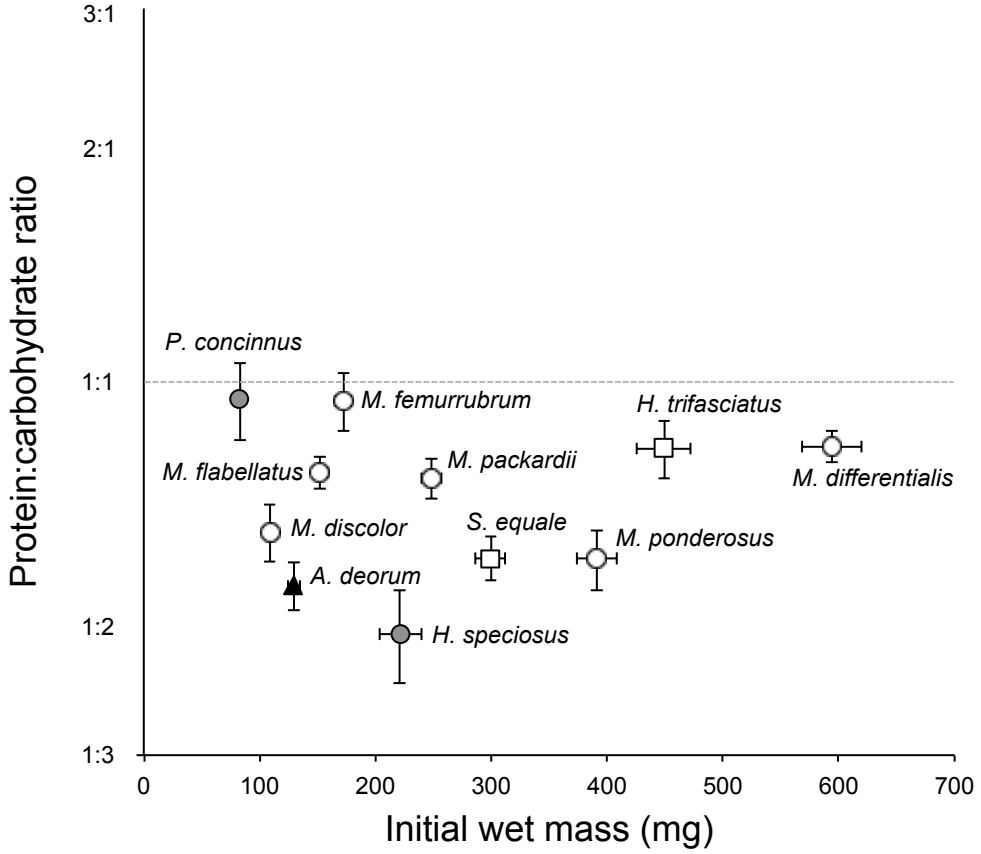

Fig. 4.5 Comparison of the protein:carbohydrate intake target ratio and initial wet mass of last instar grasshoppers for all 11 grasshopper species sampled. Protein:carbohydrate ratio is presented on a log scale to more accurately reflect the distance between ratios around a balanced 1:1 intake. Marker shape represents the grasshopper subfamily: Gomphocerinae (triangle), Oedipodinae (square), and Melanoplinae (circle). Fill color represents the functional feeding group: grass-feeder (black), forb-feeder (gray), and mixed-feeder (white).

mass: $F_{10,271}$=0.82, P =0.607) or among only mixed-feeding grasshoppers (GLM; species: $F_{7,195}$=1.44, P =0.190, initial wet mass: $F_{1,195}$=0.29, P =0.594, species×initial wet mass: $F_{7,195}$=0.99, P =0.440).

4.5 Discussion

Animals perform best when they acquire nutrients in specific self-selected ratios and amounts (Behmer and Joern 2008, Behmer 2009, Simpson and Raubenheimer 2012), and I found that the 11 species used in this study actively regulated their protein-carbohydrate intake. However, there was significant variation between species in both the observed p:c ratios, and absolute amounts of protein and carbohydrate ingested. Interestingly, all the ITs fell on the carbohydrate-biased side of nutrient space (Fig. 4.1, 4.2), in contrast to coexisting *Melanoplus* spp. from Nebraska which had p:c ratios ranging from 1.7:1 to 0.7:1 (Behmer and Joern 2008). The two species included in this study that had ITs previously determined (*M. differentialis* and *M. femurrubrum*) had ITs in my study that were shifted more towards carbohydrate intake. However, relative to other species they remained the most protein-biased similar to what was found in Nebraska (Behmer and Joern 2008). Other authors have found population-level differences in ITs of individual species and attributed the differences to body size and metabolic differences across latitude resulting from higher temperatures (Fielding and Defoliart 2008, Parsons 2011). At lower latitudes and higher temperatures ITs appear to become more carbohydrate-biased, possibly to fuel metabolic demands (Parsons 2011). Recent work has also shown that species from the same population will feed for different

ITs depending on the temperature they are restricted to (Parsons 2011), or alternatively, can post-ingestively correct for feeding on unbalanced foods by thermoregulating (Miller et al. 2009, Coggan et al. 2011). Therefore temperature at which the experiment is conducted affects results. I conducted my study at a higher temperature (day: 38°C, night: 27°C) than previous work using lower temperatures of 27-31°C (Chambers et al. 1995, Lee et al. 2002, Simpson et al. 2002, Behmer and Joern 2008). The temperatures in my experiment more accurately reflects the temperatures of feeding grasshoppers in the natural conditions at the BCNWR (F. Clissold unpublished data) and could have contributed to the more carbohydrate-biased ITs.

The consumption of nutrients is a basic necessity for all species, yet it appears that subtle differences exist even between groups of species that share a common phylogenetic ancestry, share similar body plans and gut physiology, coexist in time and space, and feed on the same host plants. This study adds to evidence reported in a past study that found significant differences in ITs at the species level (Behmer and Joern 2008). Despite all being in the family Acrididae, important behavioral and morphological differences do exist between these species (Uvarov 1977, Chapman and Joern 1990, Pfadt 2002) and I searched for patterns in the dissimilar ITs.

In both the analysis of IT absolute amounts (Fig. 4.1) and ratio (Fig. 4.2), there does not appear to be a relationship with taxonomic identity of species at the subfamily level as all taxa are interspersed in terms of their protein and carbohydrate consumption. The exception is the single gomphocerine, which had a more carbohydrate-biased intake than most other species. Although this study had a limited number of genera and species

in the Gomphocerinae and Oedipodinae, there does not appear to be any distinct taxa clustering. Further diet studies with species outside the Melanoplinae could change this finding though. The next important biologically relevant way to compare species came from differences in diet established by my analysis of gut content analysis.

Herbivores that specialize on grasses are thought to encounter fewer toxic or distasteful plant secondary metabolites (Tscharntke and Greiler 1995) but have to contend with a food that is silica-rich (McNaughton et al. 1985, Massey et al. 2006) with even less protein content than other grassland herbaceous plants, i.e. dicotelydinous forbs (Chapter II). The latter hurdle has led me to the hypothesize that grass-feeding would require a more carbohydrate-biased intake. In previous work, a grass-feeding caterpillar (Lee et al. 2003) and three grass-feeding Oedipodine grasshoppers – *Chortoicetes terminifera* (Clissold et al. 2009), *Oedipoda asiatica* (Cease et al. 2012), and *Locusta migratoria* (Chambers et al. 1995) – all regulated for carbohydrate-biased ITs. *Ageneotettix deorum*, the single gomphocerine grass-feeder in this study that accepted the artificial diet and developed to adulthood, also followed this trend. A species generally considered a mixed-feeder (Pfadt 2002), *Melanoplus differentialis*, was found to include grass as a major component of its diet at this site (Fig. 4.3). However, *M. differentialis* had a balanced IT which was significantly different from that of *A. deorum*. It seems then that, species which are restricted to grass-feeding have more carbohydrate-biased ITs, while this may not be the case for species with host plants outside the Poaceae. Work by Clissold et al. (2006) has found that in the grass-feeding *C. terminifera*, carbohydrates are a limiting nutrient due to the herbivore's poor ability to

cleave or digest through grass cell walls and access nutrients within. The carbohydrate-biased ITs of grass-specialists could therefore be due to the grasshopper's need for this limiting nutrient rather than an adaptation to mirror the nutrient content of its food (Joern and Mole 2005, Behmer 2009)

Nutrition is thought to be secondary to plant defensive chemistry when it comes to host plant selection among forb-specialists grasshoppers (Chapman et al. 1988, Bernays and Chapman 1994, Traxler and Joern 1999). However, as with the other grasshopper species, I found regulation for specific protein:carbohydrate ITs. To my knowledge the only other forb-feeding insect herbivores with measured ITs are *Manduca sexta* and *Heliothis subflexa* caterpillars which both regulated for balanced ITs (Thompson and Redak 2005, Lee et al. 2006). Between the two forb-specialists studied, the ITs were significantly different. The self-selected IT for *P. concinnus* was near a balanced ratio as we'd predict for a species feeding exclusively on relatively protein-rich dicots, however, *H. speciosus* fed for exactly the opposite: a surprisingly carbohydrate-biased target. The different ITs could be a product of their different evolutionary history (they belong to different tribes: Dactylotini and Melanoplini) or may reflect physiological adaptations to their different host plants (Fig. 4.3). *Hesperotettix speciosus* is mainly an aster-specialist while *P. concinnus* feeds on a mixture of mints and other unidentified forbs. It is possible that these ITs represent adaptations to nutrient interactions (Raubenheimer and Simpson 1990, Simpson and Raubenheimer 2001b, Behmer et al. 2002) with the iridoid glycosides, sesquiterpene lactones, and other terpenoids present in these plants (Wink 2003, Burrows and Tyrl 2012). Another

possibility is that the ITs are adaptations to limiting nutrients in these particular plants (similar to what I propose for grass-feeders) and I have found that Asteraceae generally have higher protein content than Lamiaceae species at this site (Chapter II).

The majority of species used in this study were mixed-feeding Melanoplinae and Oedipodinae. Mixed-feeding grasshopper comprise the majority of the community at the BCNWR both in terms of abundance and species richness (Chapters II, III). Species differed greatly in the absolute amounts consumed (Fig. 4.1, Appendix Table A.2) due to large differences in body size. Protein:carbohydrate ratios among the mixed feeders also differed in relation to diet (Table 4.2c, e-i), but not body size (Fig. 4.5). My gut content analysis found that many mixed feeders had broadly overlapping trophic niches (Fig. 4.3, Appendix Tables A.3, A.4) meaning these species potentially compete for host plants. In their study of Nebraskan *Melanoplus* spp. ITs Behmer and Joern (2008) suggested that coexisting generalists with overlapping diets may be partitioning nutrient niches. My findings among the mixed-feeding *Melanoplus* species fit this hypothesis. I found that IT ratios were similar between species with less overlap in diet (Table 4.2e), but IT ratios differed between species with higher diet overlap (Table 4.2f-i). The potential for nutrient niche partitioning does not seem to exist between mixed-feeding melanoplines and oedipodines. Instead I hypothesize that coexistence may be possible due to oedipodines' strong microhabitat requirement for low herbaceous cover with patches of bare ground that differs with the cover-seeking melanoplines (Otte 1984, Craig et al. 1999, Pfadt 2002).

Behmer and Joern (2008) took the idea of nutrient niche partitioning a step further and formulated three hypotheses for possible ecological implications: 1) species with similar ITs should be competing more than species with dissimilar ITs, 2) species which consumed similar absolute amounts of protein and carbohydrate would have similar relative abundances, and 3) species with extreme p:c ratios should fluctuate in abundance between years to a greater degree than those with relatively central p:c ratios. The first prediction is tested in Chapter V, and the third prediction requires long-term longitudinal studies of abundance and plant nutrient content, however I was able to address the second prediction using abundance data from Chapter III (Appendix Fig. A.1). I did find that abundance (Appendix Fig. A.1) was closely related to absolute amounts of protein and carbohydrate consumed (Fig. 4.1). For example *M. ponderosus* and *M. packardii* or *M. flabellatus* and *M.* f*emurrubrum* have similar protein and carbohydrate consumption and similar relative abundances. Conversely, *M. discolor* and *M. differentialis* have the lowest and highest absolute amounts of macronutrient consumption respectively, and the lowest and highest relative abundances. But since consumption is so closely tied with body size, this is really a factor of the well known relationship between body size and abundance (White et al. 2007). Interestingly the relationship only holds for mixed-feeding *Melanoplus* spp. but not diet specialists or mixed-feeding oedipodines. Abundance of these two is more likely tied to host plant abundance and habitat preference respectively. By diet mixing, mixed-feeders are not limited by the nutrient deficiencies or toxic plant secondary metabolites of any one host plant (Freeland and Janzen 1974, Pulliam 1975, Westoby 1978, Bernays and

Minkenberg 1997, Hagele and Rowell-Rahier 1999, Singer et al. 2002, Miura and Ohsaki 2004). Oedipodines do not follow this pattern, possibly because their abundance is closely tied to the microhabitat requirement for bare ground. These different effects of diet and behavior could be why many local size-density relationships are relatively weak (Blackburn and Gaston 1997, White et al. 2007).

Differences in ITs between populations, species, and higher taxa could be attributed to a diverse array of factors related to body size, metabolism, diet, life history traits, phylogeny, gut symbionts, and niche partitioning. This study represents another step forward in understanding patterns of nutrient regulation across species because I utilized a diverse suite of coexisting herbivores. I observed stark differences in the general distribution of ITs which may be related to temperature and latitude, functional feeding-group differences, and perhaps nutrient niche partitioning. To test whether macronutrient regulation in animals has adapted to these various factors, and is not simply a 'spandrel' (Gould and Lewontin 1979), future work will need to include nutrient regulation comparisons within a well defined phylogenetic context, compare differences in nutrient requirements to the outcome of competition experiments, and measure how different species react to nutrient manipulations of their natural food items (not artificial diets). Continued work in this area can establish why nutrient requirements vary between organisms, and how these differences affect broader ecological interactions.

CHAPTER V

TESTING THE NUTRIENT NICHE HYPOTHESIS IN GENERALIST INSECT HERBIVORES

5.1 Overview

Multiple species of generalist coexisting herbivores with overlapping diets can reach high population densities simultaneously with no adequate explanation of how coexistence is maintained when food appears to be limiting. A possible explanation, the nutrient niche hypothesis, suggests that different species could regulate for macronutrients differently, thus lessening competition for plant tissue with similar protein and carbohydrate content. I investigated this hypothesis in greenhouse microcosms using three species of generalist grasshoppers with overlapping diets, but either similar or dissimilar nutrient intake targets. Field caught grasshopper nymphs from Central Texas were caged in either monoculture, or mixed species treatments on a standard mixed plant community with one grass and two forb species. Grasshoppers in the experimental microcosms had high mortality rates in all species treatments. Forbs were the limiting resource and all species engaged in cannibalism. Analysis of cage population decline revealed competitive differences between species, but the patterns of survival gave only mixed support for the predictions of the nutrient niche hypothesis. Mortality differences were more likely linked to body size differences between species. Nutrient requirement differences have, at most, a weak effect on interspecific interactions, at least among the species tested. Nutrient requirement differences could

effect interspecific competition, but via its effect on host plant selection, and would be secondary to other factors in the field such as body size.

5.2 Introduction

Diverse groups of organisms can often be found utilizing the same foods (Hutchinson 1961, Strong 1982, Pratchett 2005). Sympatric communities of insect herbivores offer some of the best examples of this phenomenon. Herbivorous insect communities can contain dozens of species that utilize the same plant or group of plant species (Davis 1983, Kennedy and Southwood 1984, Schmitz 1998a). Under these conditions, ecological theory predicts that there should be intense competition for shared resources (Schoener 1982, Chase and Leibold 2003, Mayfield and Levine 2010, HilleRisLambers et al. 2012) and much effort has been focused on quantifying the role of competition in structuring communities of insect herbivores (Denno et al. 1995, Kaplan and Denno 2007). Many studies suggest that species can evade competition and coexist through niche partitioning (Chase & Leibold 2003). New evidence from the physiological literature has shown that insects and other animals can have finely nuanced feeding (Behmer 2009, Simpson and Raubenheimer 2012) and that differences in macronutrient needs could serve as a form of niche partitioning (Behmer and Joern 2008). In this study I test the nutrient niche hypothesis in generalist insect herbivores.

A nutrient niche would occur if individuals from different species had decreased interspecific competition by regulating for different nutrient requirement despite feeding on the same kinds of food. Behmer and Joern (2008) demonstrated that coexisting

polyphagous grasshoppers selectively feed to reach species-specific ratios of protein and carbohydrate on which performance is optimized. This blend of protein and carbohydrate is known as an intake target. Protein:carbohydrate intake targets have been demonstrated in many taxa including humans, other mammals, birds, fish, slime molds, and insects (Raubenheimer and Simpson 1997, Simpson and Raubenheimer 2001a, Simpson and Batley 2003, Behmer 2009, Felton et al. 2009, Dussutour et al. 2010). Almost all species studied selectively feed for an optimal blend of nutrients, the most important being the macronutrient ratio of protein to carbohydrate and lipids (Richter et al. 1938, Waldbauer and Friedman 1991, Raubenheimer and Simpson 1997, Simpson and Raubenheimer 2001a, Simpson and Batley 2003, Behmer 2009, Felton et al. 2009, Dussutour et al. 2010, Simpson and Raubenheimer 2012). The nutrient requirements of species have been called nutrient niches for herbivores such as caterpillars (Clancy and King 1993) and marsupials (Kinnear et al. 1979a). However, prior to the study by Behmer and Joern (2008), the idea that nutrient requirements facilitate coexistence has only been proposed in plants (Tilman 1988, Paoli et al. 2006), gut microbes (Freter et al. 1983, Chang et al. 2004), and plankton (Petersen 1975, Yoshiyama et al. 2009). These latter organisms can separate themselves in space to absorb different amounts of nutrients directly from the environment, and it is unclear whether organisms that receive nutrients packaged in other organisms (*e.g.*, herbivorous animals eating plant tissue) could reduce competition by partitioning their nutrient requirements (Simpson and Raubenheimer 2012). It may be possible that competition between species that appear to have highly overlapping diet breadths is decreased by feeding on different plant species,

different individual plants, and different plant tissues that have nutrient contents complementary to a unique macronutrient intake target.

Grasshoppers (Orthoptera: Acrididae) are an ideal system in which to investigate the possibility of nutritional niche partitioning since many species are generalist herbivores with overlapping diets. In many grassland ecosystems more than 30 grasshopper species can be found coexisting at high density. Many of these species have broadly-overlapping diets (Mulkern et al. 1969, Ueckert and M. 1971, Joern 1979a, Pfadt and Lavigne 1982, Joern 1985), co-occur in space/time at a small scale, are ecologically and phylogenetically closely related, and there is strong evidence that they compete for host plant tissue (Ritchie and Tilman 1992, Belovsky and Slade 1995, Chase 1996a, Beckerman 2000, Liu et al. 2007). These grasshopper communities are critical components of grassland ecosystems (Gibson 2009) and can be of economic concern to humans during frequent outbreaks (Hewitt and Onsager 1983, Joern and Gaines 1990, Lockwood and Lockwood 2008). Therefore, understanding how these species are able to coexist, even during outbreaks when food is severely limiting (Pfadt 1982, Watts et al. 1982), is an important question both for community ecologists and land managers.

The objective of this study was to directly test the nutrient niche hypothesis with co-occuring generalist grasshopper species. Specifically, I asked whether the outcome of competition between species could be predicted based on differences in nutrient requirements. As predicted by classic competition theory (Schoener 1983) as well as Behmer and Joern (2008), I expected species with more similar nutrient intake targets to compete more intensely for nutrient resources. To accomplish this, I first identified three

species which were found to have overlapping diet, but either similar or dissimilar protein:carbohydrate intake targets. To determine competitive dynamics between these species, I conducted a greenhouse experiment to measure survival when grasshoppers were caged with conspecifics and/or different heterospecific grasshoppers.

5.3 Methods

5.3.1 Site description

Grasshoppers used in this study were collected using a sweep net at the Balcones Canyonlands National Wildlife Refuge (BCNWR) northwest of Austin, Texas. The refuge covers parts of Burnet, Williamson and Travis Counties. The grasshopper community of the area is diverse with 56 species of Acrididae known (Appendix Table A.1), and is predominated by widespread species of the Great Plains as well as many Texas endemics. Grasshoppers were collected in areas of mixed-grass prairie and oak (*Quercus* sp.) savannah.

5.3.2 Macronutrient intake targets and competitive dynamic predictions

The species used in this experiment included *Melanoplus discolor* (Scudder), *M. differentialis* (Thomas), and *M. femurrubrum* (De Geer). These species were chosen because previous work (Chapter IV) revealed through an analysis of gut contents that all three species share host plants.

I determined the protein:carbohydrate intake targets that these species regulated for over the course of their final nymphal instar using a standardized artificial diet choice

test detailed in Chapter IV. I used field collected grasshopper nymphs from the BCNWR in June of 2012. The choice experiment used artificial diets with variable protein: carbohydrate, allowing the grasshopper to self-select its preferred macronutrient intake. Diets were prepared using the methodology of Dadd (1961) and Simpson and Abisgold (1985) with definite amounts of digestible protein and carbohydrates to calculate the % protein: % carbohydrate (henceforth p:c) ratio which each species regulated for. Diets were changed after 6 days and weighed after equilibrating in the lab for 24hrs. Upon molting into the adult stage, diets were again weighed after equilibrating in the lab, and the adult grasshoppers were weighed (wet mass) and frozen. The amount of diet consumed from each dish and the protein and carbohydrate content of each diet were used to calculate the total amounts of protein and carbohydrate consumed during the final nymphal instar. I combined treatments within species after finding similar protein:carbohydrate consumption (Chapter IV) and tested for differences between species . I compared interspecific differences in absolute amounts of protein:carbohydrate consumption using MANCOVA (Wilk's Lambda test statistic) with initial wet mass as a covariate. I also compared protein:carbohydrate intake ratio using ANOVA with log-transformed ratios. Post hoc comparisons were made using Tukey's HSD. All analyses on nutrient intake were conducted in JMP 10 (JMP 1998-2007).

This artificial diet choice test found that *M. discolor*, *M. differentialis*, and *M. femurrubrum* regulated for different absolute amount of protein and carbohydrate (Appendix Fig.A.2a, MANCOVA; Species $F_{4,158}$=6.63, P <0.001*), and had

macronutrient intake target ratios of 0.60:1 ± 0.05, 0.81:1 ± 0.04, and 0.85 ± 0.05 respectively (Appendix Fig. A.2, ANOVA; Species $F_{2,81}$=13.38, P <0.001*). *M. differentialis* and *M. femurrubrum* have similar intake target ratios (Tukey's HSD posthoc, Appendix Fig.A.2b), and therefore should be most likely to compete for plant tissue of similar nutrient composition. Conversely, *M. discolor* has a significantly more carbohydrate-biased intake than either of these species (Tukey's HSD posthoc, Appendix Fig. A.2b). Following the predictions of the nutrient niche hypothesis I hypothesized that each species would compete most against conspecifics followed by heterospecifics with a similar nutrient intake target ratio and have the least competition with heterospecifics with a dissimilar intake target. This is summarized in Fig. 5.1b.

5.3.3 Experimental protocol

To determine whether differences in nutrient intake targets correlate with levels of competition between species I assessed competition using three grasshopper species caged in small arenas in a greenhouse. Six treatments with a substitution design were used (Fig. 5.1a). These 6 treatments consisted of 3 monoculture treatments with 10 individuals of one species as well as the three unique species pair treatments with 5 individuals of each species. Each monoculture treatment was replicated in six microcosms, species pairings were replicated in eight, and a plant control of eight microcosms with no grasshoppers was used as a control for grasshopper effects on the plant community. The experiment utilized field collected 2nd and 3rd instar nymphs and followed their development over 15 days.

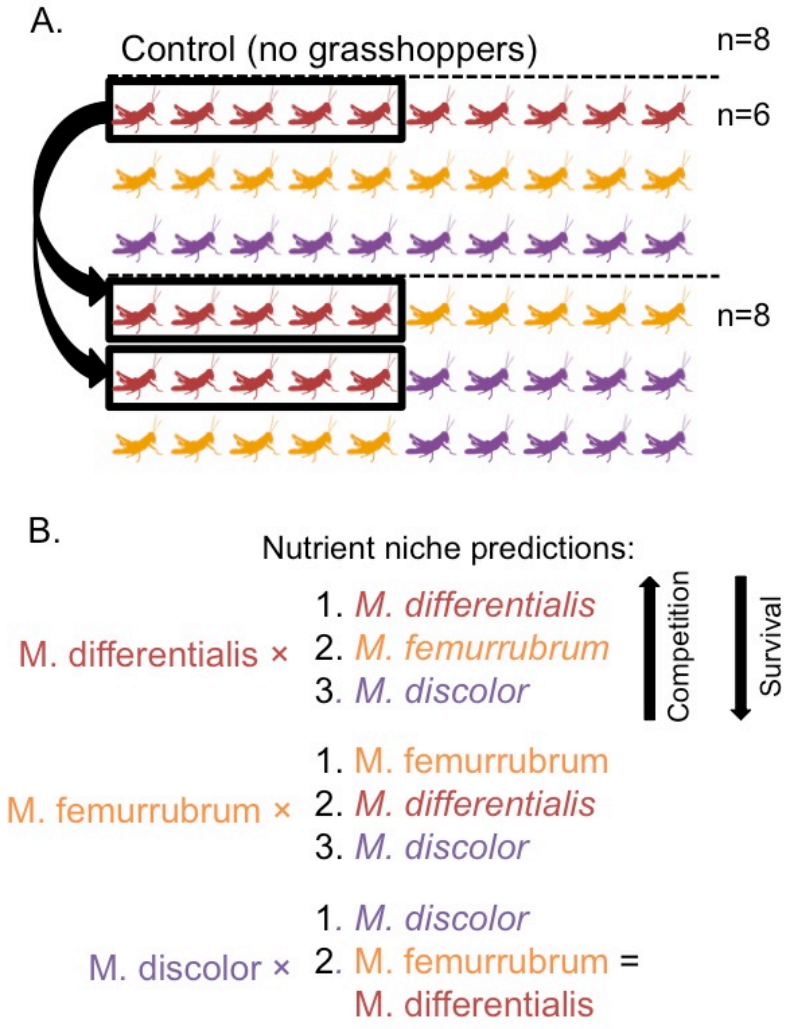

Fig. 5.1 The experimental design used in this experiment (a) and an overview of competitive predictions (b). a) Greenhouse cages were either controls with no grasshoppers present, monocultures of one of three grasshopper species, or a 50:50 mixture of two species in a substitutive design. Different species are represented by different colors. All grasshopper treatments had a total of 10 individuals. To directly compare survival between monocultures and two species treatments, I divided the surviving number of individuals in each monoculture cage by two. Comparisons for *Melanoplus differentialis* (in red) are shown with arrows between when the species is caged alone or with either of the two other species. b) An overview of competitive predictions made using the nutrient niche hypothesis (Behmer and Joern 2008). All species were expected to compete most with conspecifics (interspecific competition) due to the identical nutrient requirements. Next I expected *M. differentialis* and *M. femurrubrum* to compete most with each other because of their overlapping nutrient intake target ratios. Finally I predicted that *M. discolor* would have the least competitive interactions and highest survival when caged with either *M. differentialis* or *M. femurrubrum* do to its significantly different nutrient intake target ratio.

Fig. 5.2 Photographs of greenhouse competition cage design and plant community. a) Lateral view of a cage (45×45×73 cm) used in the greenhouse competition experiment with mixed plant community. b) Overhead view of the 27×27 cm mixed plant community used in this experiment comprised of 4-week old *Bouteloua curtipendula* (Poaceae, sideoats grama)*, Gaillardia puchella* (Asteraceae, indian blanket), and *Ratibida columnifera* (Asteraceae, Mexican hat).

Suitable numbers (160+ per species) of *M. discolor*, *M. differentialis*, and *M. femurrubrum* were collected from the BCNWR on May23, 2010. These individuals were kept in cages for a minimum of 24h and given seedling wheat, wheat bran, and romaine lettuce *ad libitum* to allow individuals injured during field collection to perish. Grasshoppers were caged in 45×45×73cm mesh microcosms (Fig. 5.2a) housed in the Borlaug Center for Southern crop improvement greenhouse (Texas A&M University). Each cage was supplied with equivalent plant communities (Fig. 5.2a) comprised of a mixture of 4-week old host plants shared by all three grasshopper species: *Bouteloua curtipendula* (Poaceae, sideoats grama)*, Gaillardia puchella* (Asteraceae, Indian blanket), and *Ratibida columnifera* (Asteraceae, Mexican hat). Seed was purchased from a source within the Edwards plateau ecoregion (Native American Seed, Junction,TX). Equivalence between the plant communities was established by mixing 40 cm^2 of *B. curtipendula* seeds, 25 cm^2 of *G. puchella* seeds, and 10cm^2 of *R. columnifera* seeds in 54×27cm plastic flats with 5 cm deep Metro Mix 900 soil (Sun Gro Horticulture Distribution Inc., Bellevue, Washington). Flats were subsequently split into 27×27cm areas of vegetation for the experiment and watered every 4 days. A mix of plant species was utilized because these species are believed to be 'true generalists' and using only one available host plant would eliminate any possibility of diet mixing, which is key for many generalist grasshoppers (Bernays and Minkenberg 1997, Miura and Ohsaki 2004, Unsicker et al. 2008, Franzke et al. 2010). The experiment ran for 15 days. During the experiment survivorship and development stage was determined for all individuals every 5 days as well as at the end of the experiment.

At the termination of the experiment I collected all grasshoppers from the cages, measured the right hind femur, and mass of all individuals. Length of the hind femur is widely used as a standard measure of grasshopper body size (Monk 1985, De Souza Santos and Begon 1987, Wall and Begon 1987, Danner and Joern 2004, Branson 2008). Grasshopper hind femur length is also correlated with functional ovarian follicles (Branson 2008) and in some cases fecundity (Atkinson and Begon 1987, Berner and Blanckenhorn 2006). At the end of the experiment all remaining plant material was clipped from the pots, and subsequently dried, sorted, and weighed. Seedlings of the two forb species were indistinguishable and are combined in analyses.

5.3.4 Statistical analyses

To determine whether survival in the experiment varied by species in the absence of interspecific competition I first compared numbers of surviving individuals in the three monoculture treatments using repeated measures ANOVA. Remaining biomass for each plant species was compared between treatments using ANOVA. When significant effects of treatment were found, I used Tukey's HSD post hoc test to determine which treatments differed. Fitness estimates were compared across treatments for each species separately, i.e. fitness estimates for individuals of species A in monoculture vs. when caged with species B vs. when caged with species C. Differences in surviving numbers of each species were analyzed using repeated measures ANOVA. Survival in monocultures was divided in half to account for the substitutive design when making comparisons between monoculture (10 individuals of 1 species) and mixed species

treatments (5 individuals of 2 different species). Differences in right hind femur length and body mass were compared using a restricted maximum likelihood (REML) mixed-model ANOVA with the random effect of cage nested within treatment. REML was utilized because mortality of grasshoppers led to an unbalanced design (SAS Institude Inc. 2012). All analyses were conducted in JMP 10 (SAS Institute, Inc.).

5.4 Results

Survival was similar for all species during the course of the experiment when caged in a monoculture (Fig. 5.3, Repeated measures ANOVA, Greenhouse-Geisser Epsilon=0.61; Species: $F_{2,15}$=0.39, P =0.682, Time: $F_{1.8, 27.3}$=136.74, P <0.001*, Time×Species Approx. $F_{3.6, 27.3}$=1.96, P =0.134). Cage populations across all treatments declined across the 15 days ending in an average of 2.3 ± 0.2 surviving individuals (76.9 ± 2.3% mortality). Among forbs, biomass was reduced to virtually 0g of dry biomass in all grasshopper treatments (Fig. 5.4a, ANOVA, Treatment: $F_{6,43}$=22.25, P <0.001*). There was no difference in final grass biomass across treatments (Fig. 5.4b, ANOVA, Treatment: $F_{6,43}$=0.91, P =0.498). Signs of cannibalism were present on corpses in 71% of the cages across all treatments

Survival of different grasshopper species was affected by the particular species it was sharing a cage. Among *M. differentialis* grasshoppers, survival decreased at a greater rate in monoculture cages when compared with *M. differentialis* caged with *M. femurrubrum* or *M. discolor* (Fig. 5.5a, Repeated measures ANOVA, Greenhouse-Geisser Epsilon=0.73; Treatment: $F_{2,19}$=6.03, P =0.009*, Time: $F_{2.2, 41.7}$=74.90,

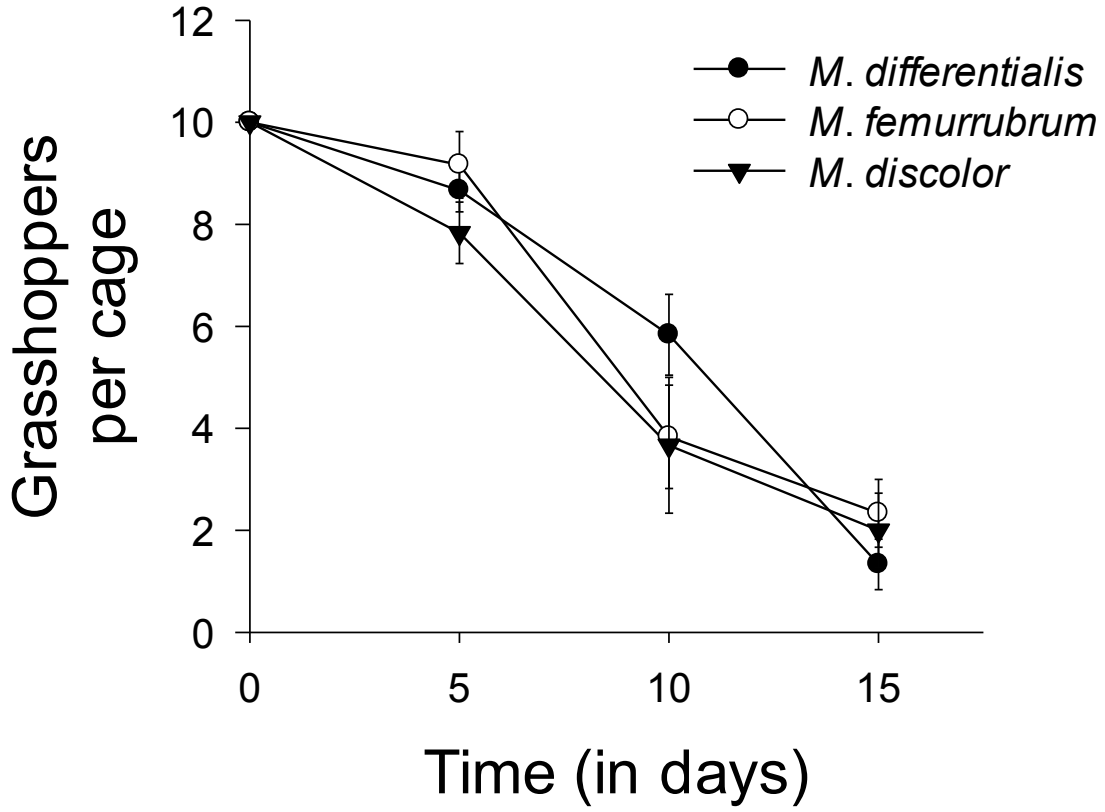

Fig. 5.3 Survival of grasshoppers in single species greenhouse cages over 15 days. Mean (±1SE) of the total number of grasshoppers per cage (n=6 per species) is given for *Melanoplus differentialis*, *M. femurrubrum*, and *M. discolor*.

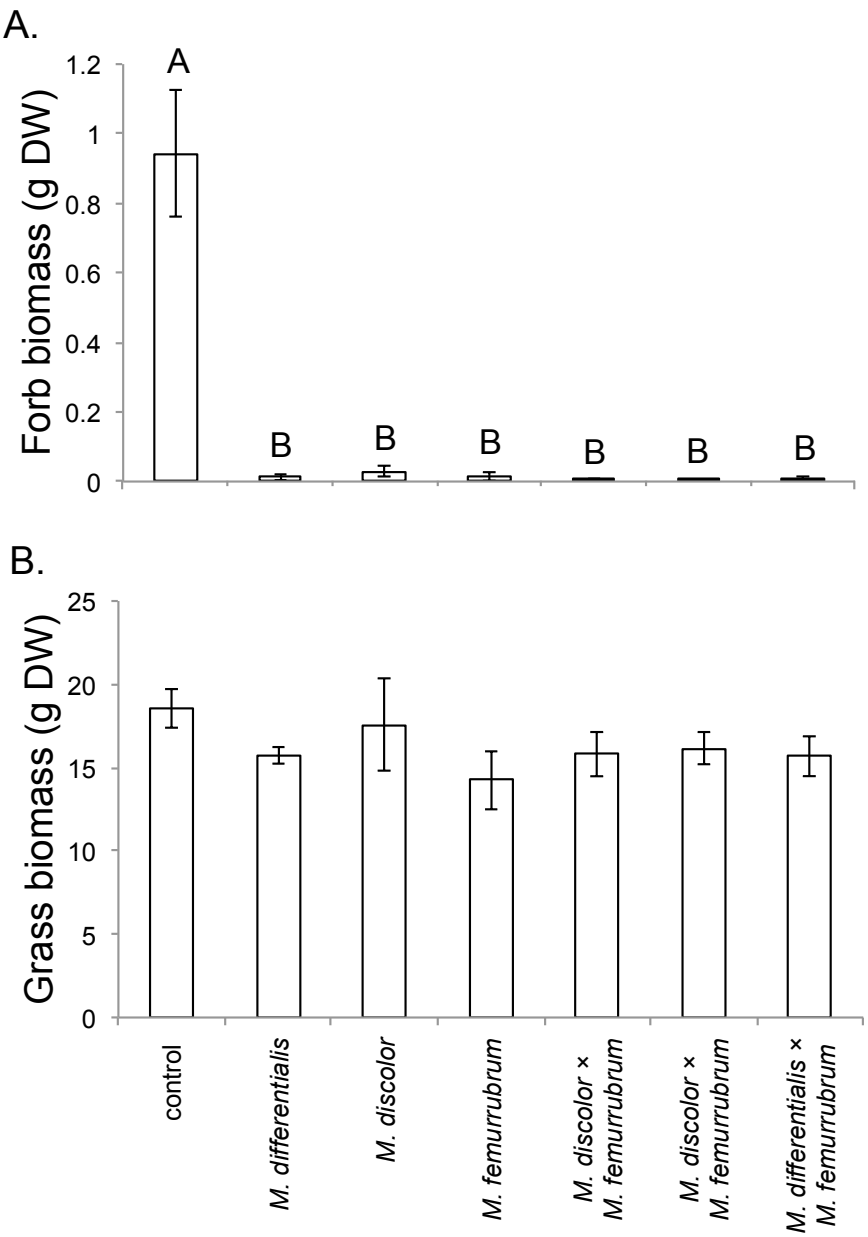

Fig. 5.4 Biomass of forbs and grass at the end of the greenhouse competition experiment. a) Dry weight biomass in grams of forbs remaining in greenhouse cages with different grasshopper treatments at the end of the experiment (15 days). This includes biomass for both *Ratibida columnifera* and *Gaillardia pulchella*. Letters indicate significant differences found in a post-hoc comparison using Tukey's HSD after a significant treatment effect was found (ANOVA, Treatment: $F_{6,43}$=22.25, P <0.001*). Mean (±1SE) b) Dry weight biomass in grams of *Bouteloua curtipendula* grass remaining in greenhouse cages with different grasshopper treatments at the end of the experiment (15 days). No significant difference across treatments was found (ANOVA, Treatment: $F_{6,43}$=0.91, P =0.498). Mean (±1SE).

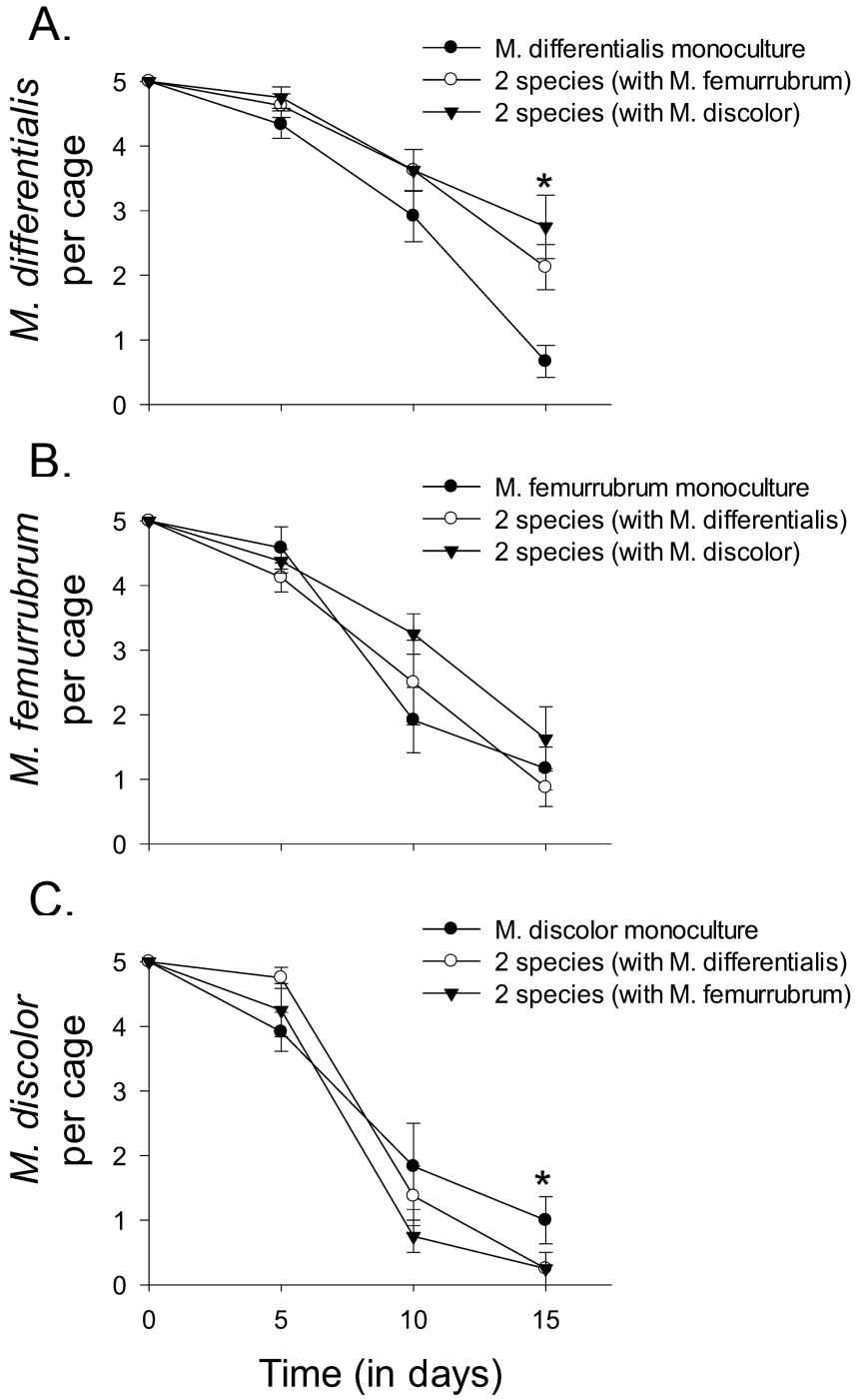

Fig. 5.5 Comparisons of grasshopper survival over the 15 day caged greenhouse competition experiment. Comparisons are made for A) *Melanoplus differentialis*, B) *M. femurrubrum*, or C) *M. discolor*.as shown in Fig. 5.1a when caged with conspecifics (black circle), or one of two heterospecifics (white circle or black triangle). Survival in single species greenhouse cages (black circles) is calculated as half the total survival (Fig. 5.3). Mean (±1SE).

P<0.001*, Time×Treatment Approx. $F_{4.4, 41.7}$=2.88, P =0.030*). There was no significant difference between *M. differentialis* survival when caged with *M. femurrubrum* or *M. discolor*.

M. femurrubrum survival was unaffected by competition treatments (Fig. 5.5b, Repeated measures ANOVA, Greenhouse-Geisser Epsilon=0.71; Treatment: $F_{2,19}$=1.53, P =0.241, Time: $F_{2.1, 40.6}$=100.83, P <0.001*, Time×Treatment Approx. $F_{4.3, 40.58}$=1.75, P =0.153). The decline in survival was similar when *M. femurrubrum* individuals were caged with conspecifics, *M. differentialis*, or *M. discolor*.

There was a marginally significant difference in how *M. discolor* survival declined between competition treatments over time (Fig. 5.5c, Repeated measures ANOVA, Greenhouse-Geisser Epsilon=0.60; Treatment: $F_{2,19}$=1.52, P =0.243, Time: $F_{1.8, 34.4}$=215.06, P <0.001*, Time×Treatment Approx. $F_{3.6, 34.4}$=2.49, P =0.066). The monoculture treatment had higher survival towards the end of the experiment compared to when *M. discolor* was caged with either *M. differentialis* or *M. femurrubrum.*

Hind femur length and wet body mass of surviving *M. differentialis* or *M. femurrubrum* were not different across competition treatments (Nested mixed-model ANOVA with REML; Hind femur length: *M. differentialis* treatment $F_{2,15.2}$=0.25, P =0.779; *M. femurrubrum* treatment $F_{2,3.6}$=0.12, P =0.890; wet body mass: *M. differentialis* treatment $F_{2,15}$=2.11, P =0.155; *M. femurrubrum* treatment $F_{2,10.7}$=0.26, P =0.776) Analyses of hind femur length and body mass could not be made for *M. discolor* due to the low numbers of survivors at the termination of the experiment.

5.5 Discussion

Even closely related organisms can differ substantially in the amounts and ratios of nutrients they require to build tissue and fuel metabolism (Simpson and Raubenheimer 1993, Behmer and Joern 2008). These differences may be byproducts, i.e. spandrels (Gould 1997), of physiological, morphological, and behavioral variations between populations. My study tested the hypothesis that differences in macronutrient requirements could represent niche partitioning (Behmer and Joern 2008). However, my greenhouse experiment found only mixed support for the nutrient niche hypothesis. Monocultures responded the same to the experimental plant community, meaning no species had an inherent advantage due to the plant species used. When comparisons were made between treatments for each species separately, significant differences in survival were found. The results for *M. differentialis* generally follow the predictions of the nutrient niche hypothesis (Fig 5.1b). Survival was lower for the monoculture treatment than either interspecific competition treatment. However, there was no significant difference between survival of *M. differentialis* when caged with *M. discolor* or *M. femurrubrum*. *Melanoplus femurrubrum* survival tended to be higher when this species was caged with *M. discolor* which would support the nutrient niche predictions (Fig 5.1b), but I was unable to detect any significant differences in this species. At the end of the experiment, *Melanoplus discolor* had higher survival in monoculture than when it was caged with either *M. differentialis* or *M. femurrubrum*. This pattern is the reverse of what would be predicted by the nutrient niche hypothesis (Fig. 5.1b).

Mortality was high, being ~77% across all treatments, which is normal among caged competition experiments with grasshoppers (Ritchie and Tilman 1992, 1993, Chase 1996b, Liu et al. 2007). Based on the preference for forbs, frequent cannibalism, and a large amount of remaining grass biomass it is likely that during the experiment grasshoppers were limited by food nutritional quality. It is tempting to assume protein and salt were limiting due to the preference for forbs and grasshopper carcasses (Simpson et al. 2006, Bazazi et al. 2008, Jonas and Joern 2008), but carbohydrates can also be limiting, especially when trapped in tough grass cell walls (Clissold et al. 2004, 2006). An analysis of protein and nonstructural carbohydrate available in these three plant species in the field (Appendix Fig. A.4) found a carbohydrate-biased nutrient space. The macronutrient ratio of *B. curtipendula* grass overlaps (Appendix Fig. A.4b) with at least one of two preferred forbs, *G. pulchella*. This indicates that other properties of the grass, possibly leaf toughness (Clissold, Sanson & Read 2004), made it unsuitable for sustaining a higher carrying capacity in the experimental cages once forbs had been eliminated. It is important to keep in mind that the macronutrient content in these plants can differ from what the insect is able to extract during digestion (Clissold et al. 2006, Clissold et al. 2010, Clissold et al. 2013).

Because of the mixed support for the effects of a nutrient niche, I explored whether there was an alternative explanation for the competitive differences I observed. The most obvious factor is body size and there were significant differences between these congeneric grasshoppers in size (Appendix Fig. A.3). Body size is well known to affect competitive dynamics (Belovsky 1986, Chase 1996a, Belovsky 1997), especially

in scenarios where exploitative competition dominates, as is the case in grasshoppers (Branson 2003). This experiment did not control for grasshopper biomass, but instead focused on number of individuals. *Melanoplus differentialis* monocultures had high mortality likely because total grasshopper biomass was highest in this treatment. *Melanoplus differentialis* survival was also highest when it was caged with the smallest grasshoppers: *M. discolor*. *Melanoplus discolor* monoculture cages had the least total grasshopper biomass, and their survival was highest in this treatment. *Melanoplus femurrubrum* is an intermediate size and therefore effects were not as strong. The competitive hierarchy found in this study though, is inherently short-term and may be different in the field when reproductive potential and other effects are included. For example, despite its competitive suppression of other species, *M. differentialis* is rare in native grassland. Conversely, the easy outcompeted *M. discolor* is the most abundant species (Chapters II, III).

Based on these findings I need to address possible problems with the nutrient niche hypothesis and revise my predictions on when could it be operating. The major criticism of the nutrient niche hypothesis is that for consumers like insect herbivores nutrients are packaged in plants and generally do not occur independently in the environment. Therefore competition still occurs at the level at the plant. Feeding by species with different macronutrient intake targets on different tissues of the same plant can still result in indirect competition via induced plant defenses, mainly plant secondary metabolites (Denno et al. 2000, Lynch et al. 2006). These plant defenses can complicate feeding decisions. I contend though that grazing "true generalist" species like

Melanoplus spp. grasshoppers would be the most likely case for insect herbivores with nutrient niches because strong effects of plant secondary metabolites are negated due to diet mixing (Freeland and Janzen 1974, Bernays and Minkenberg 1997, Hagele and Rowell-Rahier 1999, Singer et al. 2002). Effects of plant defensive chemistry seem to be negligible when grasshoppers are able to reach their IT (Simpson and Raubenheimer 2001b, Behmer et al. 2002). It is likely nutrient niche differences could effect competition under certain conditions but are probably largely inconsequential next to other ecological factors including: natural enemy effects (Schmitz 1998b), host plant populations (Belovsky and Slade 1995, Chase 1996b), competitor body size differences (Belovsky 1986, Chase 1996a, Belovsky 1997), and competitor dispersal differences (Gros et al. 2006, Picaud and Petit 2008). In addition, while the IT is the insect's feeding goal, they may not always reach it. Grasshoppers possess a range of effective post-ingestive mechanisms to correct for imbalances in what the animal has eaten (Zanotto et al. 1993, Simpson and Raubenheimer 2001b, Hahn 2005, Behmer 2009, Clissold et al. 2010, Coggan et al. 2011). The result is that herbivore performance can still be relatively high even when the animals are restricted to suboptimal diets (Behmer and Joern 2008). In summary I found that predictions based on nutrient niche partitioning alleviating competition only receive mixed support in this greenhouse cage experiment. Nutrient requirement differences probably have at most a weak effect on interspecific interactions, at least among the species tested. Nutrient requirement differences could effect interspecific competition, but via its effect on host plant selection, and would be secondary to other factors in the field. This is not to say though that interspecific

differences in nutrient regulation are inconsequential or uninteresting. In fact, by understanding why nutrient requirements and regulation differ among even closely related organisms we can better understand how feeding strategies evolve. For example, there is great interest in how nutritional differences in the hominids arose and whether they are the cause or result of different foraging strategies (Andrews et al. 1991, Oftedal et al. 1991, Leonard and Robertson 1994). Insect herbivores provide a more tractable model system to begin to understand how nutrient regulation evolves. Future studies need to investigate how nutrient requirements vary across species in a phylogenetic context to understand why requirements might differ among related species with similar diets.

CHAPTER VI

CONCLUSION

Advances in nutritional ecology, namely the development of the Geometric Framework has given investigators a standard methodology for determining how animals simultaneously regulate for important, performance affecting nutrients (Simpson and Raubenheimer 2012). Studies with a range of different organisms have found tight regulation for blends of nutrients, mostly protein and carbohydrate (Raubenheimer and Simpson 1997, Simpson and Raubenheimer 2001a, Simpson and Batley 2003, Behmer 2009, Felton et al. 2009, Dussutour et al. 2010). Interestingly the blend of protein and carbohydrate a species regulates for can vary by species (Behmer and Joern 2008, Behmer 2009). I investigated one hypothesis that could simultaneously explain why nutritional requirements in some species vary, and how generalist herbivores with overlapping diet could coexist: the nutrient niche hypothesis (Chapter I).

To address this hypothesis I first quantified how macronutrient content of plants varied in the field and attempted to link shifts across time and space to grasshopper abundance (Chapters II, III). I produced a graphical representation of the 'nutrient landscape' available to generalist grassland herbivores. The ratio of plant p:c from the field was surprisingly carbohydrate biased, with significant differences between forbs and grasses, between sampling times, and between sites. Grasshopper densities were correlated with plant macronutrient content, but relationships with the nutritional metrics differed by year. During a severe drought in 2009 the variation in plant protein and

carbohydrate shrank over the course of the summer, which was correlated to a decline in both total and grass-feeding grasshopper densities. During the wet summer of 2010 spatial variation in all grasshopper densities were negatively correlated with plant protein and protein:carbohydrate (p:c) ratio, challenging the nitrogen limitation hypothesis (McNeill and Southwood 1978, Mattson 1980, White 1993). The importance of the p:c ratio in these models reinforces that food quality can rarely be reduced to one dimension (e.g. nitrogen) but rather a balanced blend of nutrients is what is important for foraging animals (Joern and Behmer 1998, Behmer 2009, Simpson and Raubenheimer 2012).

Following two years of field sampling, and the interesting relationship with drought found in 2009 I took advantage of an even more severe seasonal drought in 2011 to experimentally manipulate water availability (Chapter III). This experimental approach gave me a much more detailed understanding of how water stress affected nutrient content in drought-hardened field plants and changes in the associated grasshopper community. Grass and forbs had different responses to the drought in terms of biomass, diversity, and macronutrient content. Grasshopper abundance and diversity was lower in water-stressed plots, which supported the findings of my previous field sampling (Chapter II), but was contrary to theory, which predicted that herbivores prefer drought-stressed plants (Lewis 1984, White 1984, Mattson and Haack 1987, Franzke and Reinhold 2011). As with the correlative analyses from 2009/2010, feeding biology mattered: different functional-feeding groups responding differently. My results demonstrate the importance of focusing on plant and insect herbivore functional groups

and provided new data for parameterizing predictive models of herbivore foraging based on macronutrient intake (Raubenheimer et al. 2009, Kearney et al. 2010, Simpson et al. 2010).

Once it was clear that plant macronutrient content was related to grasshopper abundance and community composition, I identified self-selected protein-carbohydrate intake for a suite of 11 of the coexisting grasshopper species (Chapter IV), almost doubling the number of Orthoptera species with known nutrient intake targets (Chambers et al. 1995, Simpson et al. 2002, Clissold et al. 2006, Behmer and Joern 2008, Fielding and Defoliart 2008, Boswell 2009, Goeriz Pearson et al. 2011, Parsons 2011, Cease et al. 2012). Self-selected protein-carbohydrate intake across the entire community was more carbohydrate-biased than previous studies, which could correspond to the higher temperature of the experiment (Coggan et al. 2011, Parsons 2011, Clissold et al. 2013), the lower latitude the grasshoppers were collected from (Parsons 2011), or the carbohydrate-biased nutrient landscape of the associated plant community (Chapter II). The ratio of p:c regulated for differed between species, and corresponded to differences in diet. Differences in nutrient requirements among related coexisting species with overlapping diet could be related to taxonomic and diet differences. Intake differences between these potentially competing species were not as great as in a previous study (Behmer and Joern 2008), but differences could still reflect nutrient niche partitioning as previously proposed.

Despite the differences in intake targets described using artificial diets, my cage competition study gave only mixed support for the predictions of the nutrient niche

hypothesis. Mortality differences were more likely linked to body size differences between species. Nutrient requirement differences have, at most, a weak effect on interspecific interactions, at least among the species tested. Nutrient requirement differences could effect interspecific competition, but via an effect on host plant selection, and would be secondary to other factors in the field such as body size.

Although I found only found mixed support for the nutrient niche hypothesis among the species tested, this study made some considerable advances in linking findings from the geometric framework with field-relevant biology. Future work with insect herbivores and nutritional ecology will need to focus on three focal areas. First, nutrient intake targets and macronutrient content of plants in the field need to be linked by data on how much of the plant's nutrient content is extracted and digested. Limiting factors here include the effectiveness of the insect's mandibles are at fracturing plant cell walls (Clissold et al. 2004, 2006, Clissold et al. 2009), the efficiency of the insect's digestive system (Chapman 1988, Clissold et al. 2010), and the ability of the insect to modify digestion with other factors such as body temperature (Coggan et al. 2011, Clissold et al. 2013). The second focus is associated with the first; namely understanding the complicating effects plant defenses have on nutrient regulation. Diet-mixing species like grasshoppers, and possibly other herbivores can negate the effects of toxic plant secondary metabolites through adequate macronutrient regulation (Raubenheimer and Simpson 1990, Raubenheimer 1992, Behmer et al. 2002). In other cases, plant secondary metabolites can change the amount of available macronutrient content in the plant (DeGabriel et al. 2008, Wallis et al. 2010). Finally, and most importantly, a study on the

evolution of macronutrient intake targets could determine which factors influence this trait. One of the most powerful ways to address which factors affect the evolution of nutritional intake is a comparative physiological study in a phylogenetic context. Many comparisons of nutrient regulation emphasize unrelated taxa or only compare a pair of species and therefore can be confounded by phylogenetic distance between species (Garland et al. 2005). Researchers are now increasingly calling for better phylogenetic control in comparative studies (Horn et al. 2006, German et al. 2010, Karasov et al. 2011). A comprehensive study of macronutrient regulation adaptations to diet would lead to a transformative understanding of why species differ in foraging and consumption behavior.

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APPENDIX

Table A.1 Acrididae of the Balcones Canyonlands NWR, Texas with habitat preference and diet categorization based on personal observations, data from Chapter IV, and the literature (Capinera and Sechrist 1981, Richman et al. 1993, Pfadt 2002).

		General Habitat				Diet			Abundance
		Fields	Bare patches	Riparian	Wooded	Trees/ Shrubs	Forbs	Grasses	
Cyrtacanthacridinae	*Schistocerca americana*	X				X	X		U
	Schistocerca nitens	X				X	X		U
	Schistocerca obscura			X	X	X	X		R
	Schistocerca lineata			X	X	X			U/locally C
Melanoplinae	*Campylacantha olivacaea*	X					X		F
	Hesperotettix speciousus	X		X			X		C
	Hesperotettix viridis	X					X		U
	Melanoplus angustipennis	X					X	X	U
	Melanoplus bispinosus	X					X	X	R
	Melanoplus differentialis	X					X	X	U
	Melanoplus discolor	X					X	X	C
	Melanoplus confusus	X	X				X	X	F
	Melanoplus femurrubrum	X					X	X	C
	Melanoplus flabellatus	X					?	?	C
	Melanoplus keeleri	X					X	X	R
	Melanoplus packardii	X					X	X	F
	Melanoplus ponderosus	X			X		X	X	F
	Melanoplus punctulatus				X	X			R
	Melanoplus sanguinipes			X			X	X	R
	Melanoplus tuberculatus	X					?	?	F
	Paraidomena punctata	X		X			?		C
	Phaedrotettix concinnus	X		X			X		C
	Phaulotettix eurycercus	X					?	?	F
Gomphocerinae	*Acrolophitus hirtipes*			X			X		R
	Ageneotettix deorum	X						X	C
	Amblytropidia mysteca	X		X	X			?	U
	Boopedon auriventris	?			?			?	R
	Boopedon gracile	X						X	C
	Dicromorpha viridis			X				X	U
	Eritettix abortivus	X	X					X	U
	Mermiria bivitatta	X						X	C
	Opeia obscura	X						X	U
	Orphulella speciosa	X						X	U
	Phlibostroma quadrimaculata	X						X	R
	Psoloessa texana	X	X					X	U
	Syrbula admiralis	X						X	C
Oedipodinae	*Arphia conspersa*	X	X					X	R
	Arphia simplex	X	X					X	C
	Arphia xanthoptera	X			X			X	R
	Chortophaga viridifasciata	X	X					X	F
	Dissosteira carolina			X			X	X	U
	Encoptolophus costalis	X	X					X	C
	Encoptolophus subgracilis			X			X	?	U
	Hadrotettix trifasciatus		X				X	X	C
	Hippiscus ocelote		X	X				X	C
	Hippopedon capito		X					?	R
	Lactista aztecus		X					X	U
	Psinidia amplicornis		X				?		U
	Spharagemon equale		X				X	X	C
	Spharagemon cristatum		X					?	U
	Tracyrachys kiowa		X					X	C
	Trimerotropis maritima		X				?		R
	Trimerotropis pallidipennis		X				X	X	R
	Trimerotropis pistrinaria		X				X	X	R
	Xanthippus corallipes		X					X	U

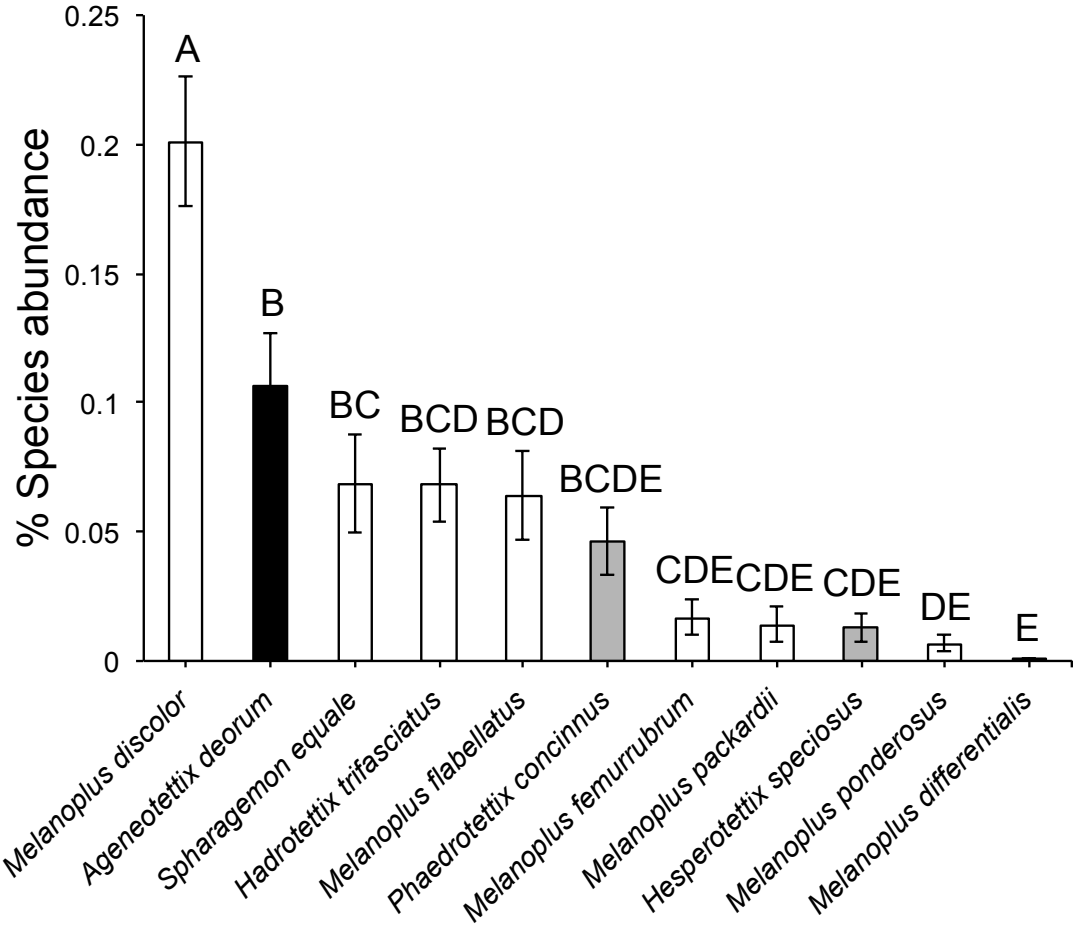

Fig. A.1 Mean relative species abundance (%) of select grasshopper species included in nutritional intake study based on field samples from the Balcones Canyonland NWR. Other grasshopper taxa are not shown. Data from May-August 2011 (across 4 sites). Relative species abundance was calculated per plot using all grasshopper taxa found during visual inspections of $1m^2$ plots distributed across multiple sites. See Chapter III for full experimental design. Letters above each species' relative abundance reflect significant differences inferred from post hoc analysis (Tukey's HSD) after a significant overall species effect was found (ANOVA: $F_{10,1298}$= 17.88, P<0.001*) using arcsine square root transformed relative abundances. There was no significant effect of time or time*species on the relative abundance of species during the months sampled. Error bars represent standard error. Bars are color coded by functional feeding group (black: grass-feeder, gray: forb-feeder, white: mixed-feeder).

Table A.2 Comparison of protein-carbohydrate intake targets (corrected for size) between all 11 grasshopper species. Comparisons were made using specified contrast statements after a significant overall species effect was found (MANOVA: Wilk's Lambda $F_{20,570}$=12.45, p<0.001*). The bonferroni corrected significant alpha = 0.0009.

Species comparison		Exact F	Prob>F	Sig.
Ageneotettix deorum	*Hadrotettix trifasciatus*	5.46	0.0047	
Ageneotettix deorum	*Hesperotettix speciosus*	1.24	0.2906	
Ageneotettix deorum	*Melanoplus differentialis*	53.88	<0.0001	*
Ageneotettix deorum	*Melanoplus discolor*	19.39	<0.0001	*
Ageneotettix deorum	*Melanoplus femurrubrum*	21.85	<0.0001	*
Ageneotettix deorum	*Melanoplus flabellatus*	25.44	<0.0001	*
Ageneotettix deorum	*Melanoplus packardii*	42.66	<0.0001	*
Ageneotettix deorum	*Melanoplus ponderosus*	17.80	<0.0001	*
Ageneotettix deorum	*Phaedrotettix concinnus*	8.58	0.0002	*
Ageneotettix deorum	*Spharagemon equale*	15.38	<0.0001	*
Hesperotettix speciosus	*Hadrotettix trifasciatus*	7.69	0.0006	*
Hesperotettix speciosus	*Melanoplus differentialis*	19.39	<0.0001	*
Hesperotettix speciosus	*Melanoplus discolor*	3.48	0.0321	
Hesperotettix speciosus	*Melanoplus femurrubrum*	19.21	<0.0001	*
Hesperotettix speciosus	*Melanoplus flabellatus*	19.22	<0.0001	*
Hesperotettix speciosus	*Melanoplus packardii*	31.87	<0.0001	*
Hesperotettix speciosus	*Melanoplus ponderosus*	10.31	<0.0001	*
Hesperotettix speciosus	*Phaedrotettix concinnus*	10.13	<0.0001	*
Hesperotettix speciosus	*Spharagemon equale*	9.34	0.0001	*
Melanoplus differentialis	*Hadrotettix trifasciatus*	28.66	<0.0001	*
Melanoplus differentialis	*Melanoplus discolor*	27.92	<0.0001	*
Melanoplus differentialis	*Melanoplus femurrubrum*	8.22	0.0003	*
Melanoplus differentialis	*Melanoplus flabellatus*	6.38	0.0020	
Melanoplus differentialis	*Melanoplus packardii*	0.81	0.4453	
Melanoplus differentialis	*Melanoplus ponderosus*	14.78	<0.0001	*
Melanoplus differentialis	*Phaedrotettix concinnus*	24.70	<0.0001	*
Melanoplus differentialis	*Spharagemon equale*	15.04	<0.0001	*
Melanoplus discolor	*Hadrotettix trifasciatus*	6.13	0.0025	
Melanoplus discolor	*Melanoplus femurrubrum*	10.43	<0.0001	*
Melanoplus discolor	*Melanoplus flabellatus*	8.88	0.0002	*
Melanoplus discolor	*Melanoplus packardii*	19.95	<0.0001	*
Melanoplus discolor	*Melanoplus ponderosus*	4.47	0.0122	
Melanoplus discolor	*Phaedrotettix concinnus*	6.74	0.0014	
Melanoplus discolor	*Spharagemon equale*	2.35	0.0968	
Melanoplus femurrubrum	*Hadrotettix trifasciatus*	6.49	0.0017	
Melanoplus femurrubrum	*Melanoplus flabellatus*	1.41	0.2464	
Melanoplus femurrubrum	*Melanoplus packardii*	7.96	0.0004	*
Melanoplus femurrubrum	*Melanoplus ponderosus*	11.35	<0.0001	*
Melanoplus femurrubrum	*Phaedrotettix concinnus*	4.26	0.0150	
Melanoplus femurrubrum	*Spharagemon equale*	7.42	0.0007	*
Melanoplus flabellatus	*Hadrotettix trifasciatus*	10.51	<0.0001	*
Melanoplus flabellatus	*Melanoplus packardii*	4.64	0.0104	
Melanoplus flabellatus	*Melanoplus ponderosus*	6.46	0.0018	
Melanoplus flabellatus	*Phaedrotettix concinnus*	8.10	0.0004	*
Melanoplus flabellatus	*Spharagemon equale*	3.78	0.0240	
Melanoplus packardii	*Hadrotettix trifasciatus*	24.78	<0.0001	*
Melanoplus packardii	*Melanoplus ponderosus*	8.68	0.0002	*
Melanoplus packardii	*Phaedrotettix concinnus*	21.57	<0.0001	*
Melanoplus packardii	*Spharagemon equale*	9.35	0.0001	*
Melanoplus ponderosus	*Hadrotettix trifasciatus*	15.51	<0.0001	*
Melanoplus ponderosus	*Phaedrotettix concinnus*	15.01	<0.0001	*
Melanoplus ponderosus	*Spharagemon equale*	0.99	0.3727	
Phaedrotettix concinnus	*Hadrotettix trifasciatus*	0.32	0.7269	
Phaedrotettix concinnus	*Spharagemon equale*	10.00	<0.0001	*
Hadrotettix trifasciatus	*Spharagemon equale*	10.73	<0.0001	*

Table A.3 Grasshopper diets as determined by crop content analysis. Each food category is represented by frequency and proportion (%) at which it occurred among grasshopper crops pooled for each species. Dicots with no distinguishing feature, mainly no trichomes, are combined ('Undet. Dicots/ smooth forbs'). Based on my reference plant library this could potentially include plants within the Asteraceae, Euphorbiaceae, Fabaceae, Gentianaceae, and Rubiaceae. Sample size is given (n). Unidentified plants have been numbered with the individual grasshopper sample number that they were first found in.

	species	*Ageneotettix deorum*		*Hesperotettix speciosus*		*Melanoplus differentialis*		*Melanoplus discolor*		*Melanoplus femurrubrum*		*Melanoplus flabellatus*	
	n	15		14		15		15		15		15	
Plant family	Food item	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.
	Empty crop	0.07	1	0.17	2							0.15	2
	Unidentified matter					0.20	3	0.07	1			0.15	2
	Arthropod parts	0.16	5										
Apocynaceae	*Asclepias*												
Asteraceae	undet. Asteraceae				3					0.07	1	0.00	1
Asteraceae	Asteraceae flower				2			0.07	1	0.12	3		
Asteraceae	Forb sp. 149							0.07	1				
Asteraceae	*Gaillardia*							0.07	1				
Asteraceae	*Guttierrezia*			0.08	1					0.07	1		
Asteraceae	*Ratibida*			0.25	3								
Asteraceae	Forb sp. 166									0.07	1		
Boraginaceae	*Heliotropium*							0.02	1	0.02	1		
Convulvulaceae	*Evolvulus*							0.16	3	0.07	2	0.04	1
Euphorbiaceae	*Croton*												
Fabaceae	*Desmanthus*												
Lamiaceae	Forb sp. 300							0.07	1				
Lamiaceae	*Hedioma*							0.07	1	0.03	1		
Lamiaceae	*Monarda*							0.10	2	0.20	3		
Lamiaceae	*Salvia*												
Lamiaceae	*Scutellaria*							0.07	1				
Malvaceae	*Sida*					0.03	1					0.08	1
Plantaginaceae	*Plantago*												
Poaceae	*Aristida*	0.42	6					0.02	1	0.07	2		
Poaceae	*Bothrochloa*					0.07	1						
Poaceae	Poaceae	0.35	6			0.40	6	0.02	1	0.06	2	0.05	2
Poaceae	*Sorghum*	0.07	1			0.07	1						
Polygalaceae	*Polygala alba*									0.07	1		
Polygalaceae	*Polygala lindheimeri*												
Solanacaeae	*Chamaesaracha*												
undet. dicots	Smooth forb			0.17	3			0.14	4			0.14	2
undet. dicots	Forb sp. 333												
undet. dicots	Forb sp. 447							0.07	1				
undet. dicots	Forb sp. 394											0.18	3
Verbenaceae	*Phyla*									0.03	1		
Verbenaceae	*Verbena*					0.17	3			0.13	3	0.35	6
Verbenaceae	*Verbena* flower					0.07	1						

Table A.3 Continued

	species	*Melanoplus packardii*		*Melanoplus ponderosus*		*Phaedrotettix concinnus*		*Hadrotettix trifasciatus*		*Spharagemon equale*	
	n	15		10		15		18		7	
Plant family	Food item	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.
	Empty crop										
	Unidentified matter	0.07	1	0.10	1	0.07	1	0.17	3		
	Arthropod parts					0.07	3	0.09	3		
Apocynaceae	*Asclepias*							0.13	3		
Asteraceae	undet. Asteraceae	0.07	1	0.20	2	0.07	1				
Asteraceae	Asteraceae flower	0.07	1	0.10	1						
Asteraceae	Forb sp. 149	0.07	1								
Asteraceae	*Gaillardia*										
Asteraceae	*Guttierrezia*										
Asteraceae	*Ratibida*										
Asteraceae	Forb sp. 166										
Boraginaceae	*Heliotropium*	0.02	1					0.08	2		
Convulvulaceae	*Evolvulus*	0.16	4					0.10	3	0.11	3
Euphorbiaceae	*Croton*			0.10	1						
Fabaceae	*Desmanthus*	0.07	1					0.01	1		
Lamiaceae	Forb sp. 300	0.17	3								
Lamiaceae	*Hedioma*	0.03	1			0.13	2	0.02	1		
Lamiaceae	*Monarda*					0.07	1	0.06	2	0.21	2
Lamiaceae	*Salvia*			0.03	1	0.20	3	0.11	2		
Lamiaceae	*Scutellaria*	0.03	1					0.03	1		
Malvaceae	*Sida*			0.03	1			0.07	2		
Plantaginaceae	*Plantago*	0.09	2								
Poaceae	*Aristida*			0.10	1						
Poaceae	*Bothrochloa*										
Poaceae	Poaceae	0.09	3	0.05	3			0.01	1	0.01	1
Poaceae	*Sorghum*										
Polygalaceae	*Polygala alba*										
Polygalaceae	*Polygala lindheimeri*	0.01	1					0.06	1		
Solanacaeae	*Chamaesaracha*	0.03	1								
undet. dicots	Smooth forb			0.10	1	0.33	6	0.04	1	0.52	4
undet. dicots	Forb sp. 333	0.03	1			0.07	1	0.06	1		
undet. dicots	Forb sp. 447										
undet. dicots	Forb sp. 394										
Verbenaceae	*Phyla*										
Verbenaceae	*Verbena*			0.20	2					0.14	1
Verbenaceae	*Verbena* flower										

Table A.4 Diet niche overlap (Pianka index) among different co-occurring grasshopper species as determined by gut content analysis. The Pianka index varies from 0-1 with 0 being no niche overlap, and 1 being complete resource-use overlap. Underlined values indicate Pianka index values of >0.6 which indicates substantial resource-use overlap (Capello et al. 2012).

	H. speciosus	*M. differentialis*	*M. discolor*	*M. femurrubrum*	*M. flabellatus*	*M. packardii*	*M. ponderosus*	*P. concinnus*	*H. trifasciatus*	*S. equale*
A. deorum	0.0	0.83	0.22	0.39	0.23	0.34	0.65	0.11	0.23	0.17
H. speciosus		0.00	0.52	0.60	0.31	0.38	0.55	0.32	0.07	0.23
M. differentialis			0.21	0.56	0.55	0.32	0.79	0.00	0.13	0.24
M. discolor				0.65	0.50	0.82	0.55	0.82	0.70	0.86
M. femurrubrum					0.51	0.77	0.84	0.32	0.44	0.42
M. flabellatus						0.27	0.61	0.45	0.23	0.68
M. packardii							0.57	0.50	0.70	0.63
M. ponderosus								0.29	0.28	0.38
P. concinnus									0.69	0.74
H. trifasciatus										0.61

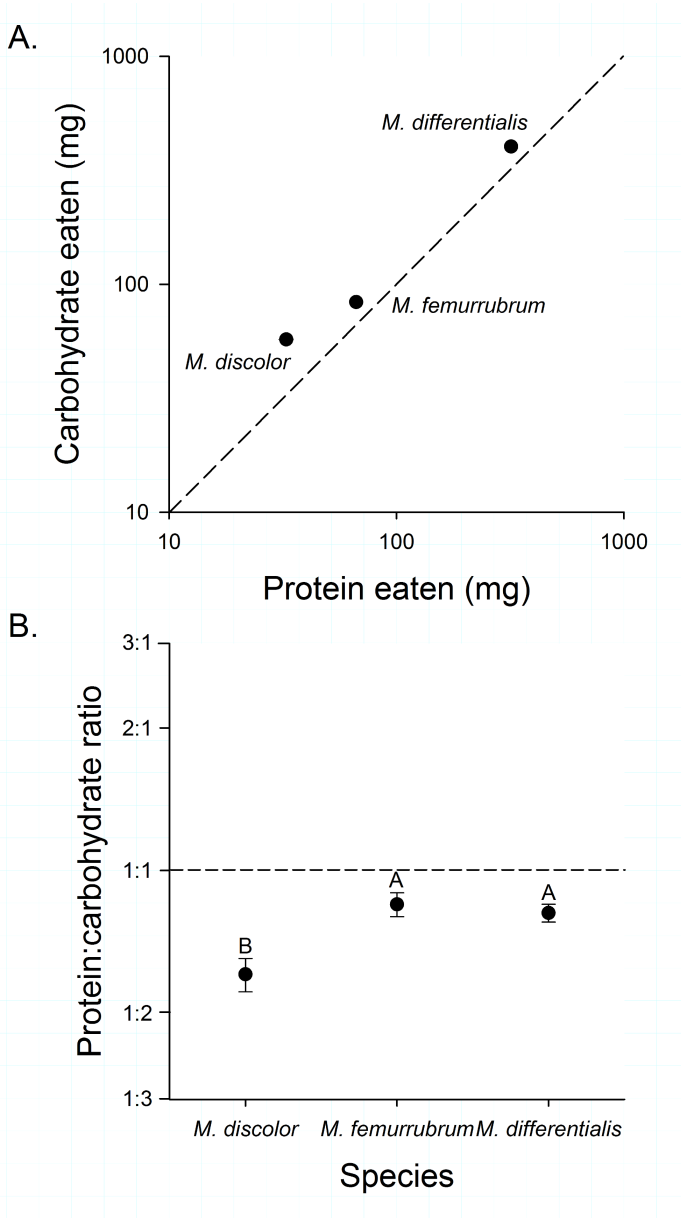

Fig. A.2 Total protein and carbohydrate (a) and the protein:carbohydrate ratio (b) consumed by *Melanoplus discolor*, *M. femurrubrum*, and *M. differentialis* grasshoppers from Central Texas. Intake targets shown reflect the amount of macronutrients consumed in an artificial diet choice experiment during entire final nymphal instar. Total protein and carbohydrate axes in 'A' are presented on a log scale to account for differences in consumption based on body size. Protein:carbohydrate ratio in 'B' is presented on a log scale to more accurately reflect the distance between ratios around a balanced 1:1 intake. Standard error bars are plotted in both A and B (not visible in A due to scale). Species not connected by the same letter in B are significantly different (Tukey's HSD posthoc test).

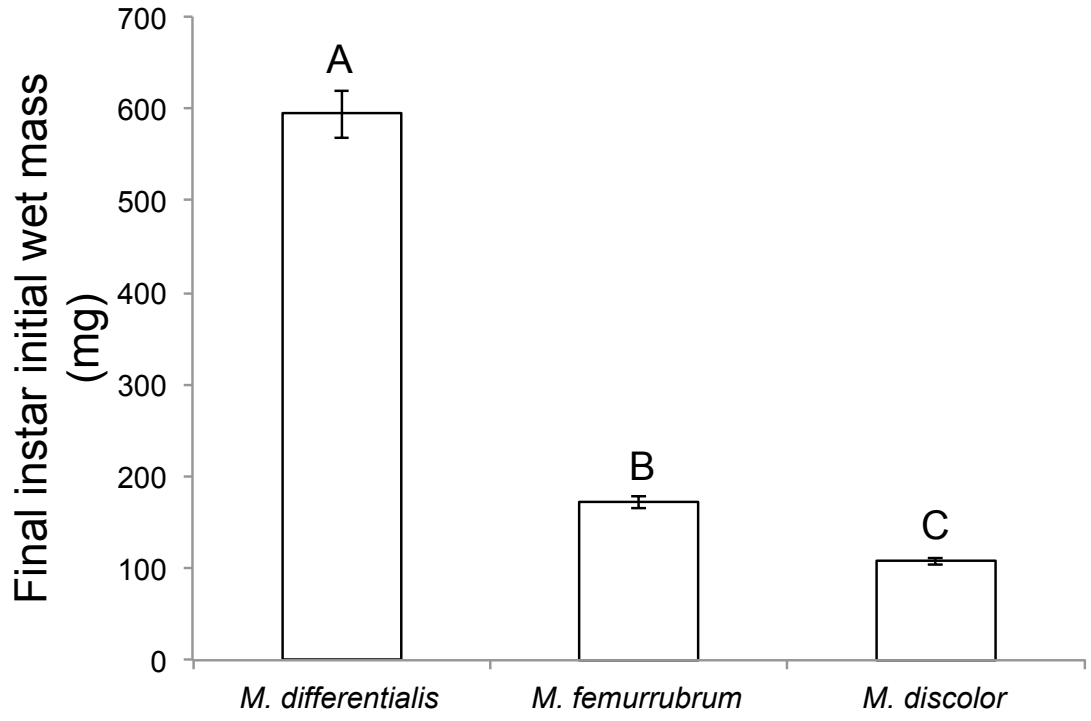

Fig. A.3 Wet mass comparison of last instar *Melanoplus* grasshoppers. *Melanoplus differentialis* (n=26), *M. femurrubrum* (n=26), and *M. discolor* (n=34) nymphs were weighed at the beginning of their final nymphal instar. Grasshoppers were collected from the Balcones Canyonlands NWR as 2-3rd instar nymphs and reared on organic romaine lettuce and seedling wheat as part of the artificial diet experiment (Chapter IV). Letters represent significantly different groups as assigned by Tukey's HSD posthoc test following finding a significant effect of species (ANOVA, species: $F_{2,83}$=335.72, p<0.001*).

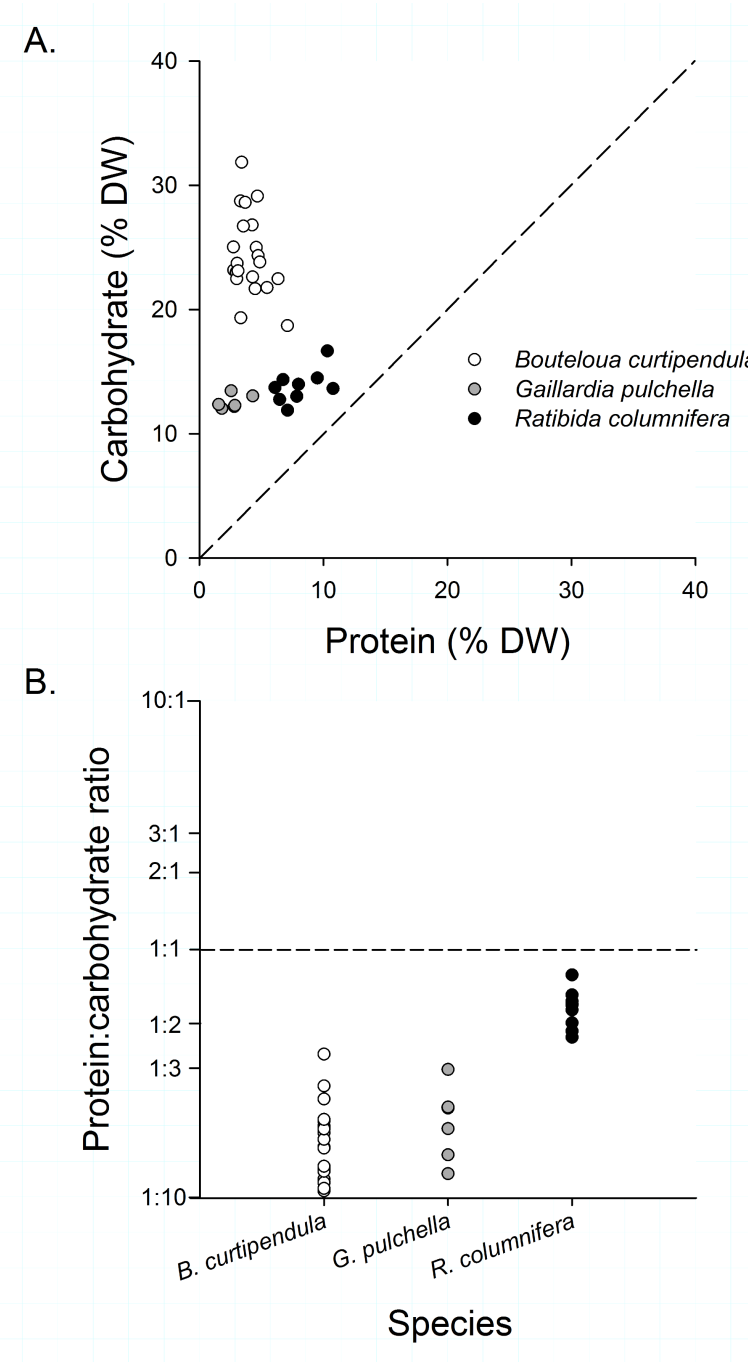

Fig. A.4 Absolute amounts of protein and nonstructural carbohydrate content (a) and the protein:carbohydrate ratio (b) of individual field-collected *Bouteloua curtipendula* (Poaceae, sideoats grama)*, Gaillardia puchella* (Asteraceae, indian blanket), and *Ratibida columnifera* (Asteraceae, Mexican hat) determined in Chapter II. Macronutrients (% dry weight) were quantified using flash-frozen, lypholized, and milled plant samples using analyses detailed in Chapter II.